INTERACTION OF *RHIZOBIUM JAPONICUM* WITH SOYBEAN ISOLINES CARRYING UNIQUE GENES WHICH AFFECT NODULATION AT THE Rj_1 LOCUS

By

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To Four Teachers

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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The soybean genotype rj_1rj_1 conditions the inability to nodulate with most strains of *Rhizobium japonicum*. Certain strains, termed overcoming strains, form a few nodules on plants grown in hydroponic culture. The effect of temperature on the number of nodules per plant and the nodulation pattern was determined for the cultivar Clark (Rj_1Rj_1) and its isoline Clark-rj_1 (rj_1rj_1). The temperature treatments, 22 C, 27 C, and 32 C, had a statistically significant effect on the number of nodules for both genotypes. The percentage of Clark-rj_1 plants nodulated by overcoming strains was 44% at 22 C, 23% at 27 C, and 4% at 32 C for 120 plants tested. The nonovercoming strain 110 did not nodulate Clark-rj_1 at any temperature. Ninety-eight percent of 270 Clark plants tested, including all strains and treatments, were nodulated. Frequency plots

were generated for each isoline x strain combination for each temperature. These indicated the number of nodules produced at locations on the primary root relative to the location of the root tip which was marked at the time of inoculation. Plots for combinations of Clark with each strain showed a peak near the root tip mark for plants grown at 22 C or 27 C. The frequency plots for Clark plants grown at 32 C were flattened and indicated a downward displacement of nodulation on the primary root.

The adsorption of overcoming strain 94 and nonovercoming strain 110 to roots of Clark and Clark-$\underline{rj}_1$ was tested. After 2 hr inoculation, approximately 100 bacterial cells were bound per plant, irrespective of strain. The rank of isoline x strain combinations for the number of bacteria bound to roots was opposite to that for nodule number.

The roots of both plant types were examined by light microscopy and scanning electron microscopy 10 d after inoculation with strain 94 or 110. Curled root hairs with infection threads were formed on Clark in response to either bacterium, but were not observed on Clark-$\underline{rj}_1$. When Clark-$\underline{rj}_1$ plants were inoculated at a high inoculum concentration, perforation in the epidermis was apparent, suggesting a potential infection pathway.

CHAPTER ONE
INTRODUCTION

The symbiosis of soybean and the bacterium Rhizobium japonicum depends, even in early stages of its initiation, on contributions from both the plant and the bacterium. They interact in a coordinated, multi-step infection process to form root nodules. The bacteria inhabit cells of the nodule, where they fix atmospheric nitrogen, which is unusable to the plant, to ammonium, a plant nutrient. The rhizobia in turn derive sustenance from the plant in the form of translocated nutrients. The series of events that transpire during the infection process has been studied by microscopy, by induced genetic change in the bacterium, and by biochemical and microbiological examination. Some steps leading to nodule formation have been described, but the relative contributions of the plant and the bacterium to the inception of a nodule are not well understood. The literature that describes the infection process is reviewed in Chapter Two. Studies are described in which genetic changes are induced in Rhizobium and the effects of the changes are correlated with the interaction phenotype with the host. The contribution of studies of plants with altered symbiotic phenotype also is described.

The soybean genotype, $\underline{rj_1}\underline{rj_1}$, conditions resistance of soybean to most strains of R. japonicum. A few strains, called the "overcoming strains," have the ability to overcome the $\underline{rj_1}$-resistance and form a few nodules on $\underline{rj_1}\underline{rj_1}$-plants. In this study the soybean cultivar Clark and a line near-isogenic to it, Clark-$\underline{rj_1}$ that carries the genes $\underline{rj_1}\underline{rj_1}$, were used. Chapters Three through Five are reports on aspects of the phenotypic expression of the symbiotic interaction of soybean isolines and strains of R. japonicum. Chapter Three describes the effect of temperature on the differential nodulation response of the soybean isolines. Chapter Four presents an evaluation of the role of bacterial adsorption as a specific determinant of differential nodulating ability. Chapter Five includes a report of the interaction phenotype at the cellular level after inoculation of seedlings with an overcoming or a nonovercoming strain. Conclusions about some aspects of the interaction of strains of R. japonicum with the soybean isolines are summarized in Chapter Six. The appendices include a short description of the plasmid complement of strains of R. japonicum as determined by gel electrophoresis and a report of an evaluation of procedures which had been described to induce high-frequency mutations in symbiotic functions.

CHAPTER TWO
REVIEW OF NODULATION SPECIFICITY AND MODE OF INFECTION BY *RHIZOBIUM JAPONICUM* OF SOYBEAN LINES WITH NODULATION RESTRICTIVE GENOTYPES

Introduction

Soybean has had the largest increase in acreage for any crop in American history. In 1930, 1 million acres produced about 14 million bushels; in 1980 more than 70 million acres produced over 2.25 billion bushels (Sundquist 1981). Over the past 60 years there has been about one-quarter bushel per acre increase in yield per year. This is due in great measure to increased understanding through research on soybean production. Included is the understanding and manipulation of its symbiosis with root-nodule bacteria (Hanson 1981, Weber 1981). Weber (1981) has estimated the market value for the combined nitrogen produced in the United States by the legume-*Rhizobium* symbiosis at $5 billion a year.

The value of the symbiosis and the impact of its improvement are clear, but the exact nature of the relationship of the bacterium to the plant and how best to manipulate that relationship for greater productivityare still unclear. The subtle interchange of signals between plant and microsymbiont that leads to establishment and maintenance of the complex partnership has largely remained

a mystery, in spite of considerable effort to unravel its intricacies. More is known about the genetic and physiological factors essential for the bacterium in this relationship because of the greater ease with which these elements can be manipulated in the relatively simpler organism. Although a few genotypes are known to produce qualitative changes in the phenotype of the interaction, our knowledge of the role of the plant is limited mostly to general observations of the effect of genetic constitution on quantitative aspects of the symbiosis.

To replace the broad, nonanalytical descriptions of the relationship, Vincent (1980) proposed terminology that defines specific steps in the establishment of the partnership. His terms are defined as specific phenotypes meant to be applicable to most legume-Rhizobium symbioses, although there is no provision for rhizobia that infect by means other than through root hairs. The terms pertinent to early infection processes will be used in this discussion, and are as follows (derivation are indicated): Roc = Root colonization, Roa = Root adhesion, Hac = Hair curling, and Inf = Infection thread formation. Two common terms not described by Vincent will be used for events representative of the mature symbiosis. The term Nod (= Nodule formation) will be used to describe the formation of macroscopic nodules. The term Fix (= Fixation of nitrogen) will be used, rather than Nif, to avoid confusion with bacterial genetic nomenclature.

This review first examines the rhizobia and their contribution to the infection of legumes. The taxonomy of the Rhizobiaceae is discussed in light of its important role in defining research directions and its implications for better understanding of genetic data. Experimental genetic manipulation of the rhizobia is included to provide a background for discussion of the research presented in subsequent chapters. The literature which describes the phenotype of interaction of the bacterium and plant at the cellular level is reviewed. Plant genotypes known to have qualitative effects on the early stages of nodulation also are described.

"Cross-inoculation Groups" and Rhizobium Taxonomy

The taxonomy of *Rhizobium* is based on the range of host plants nodulated. Strains with similar host-range have been given species status. This idea was formalized in the landmark monograph of Fred, Baldwin, and McCoy in 1932. The authors of this monograph expounded the concept of "the cross-inoculation group." This amounted to an exhaustive list of the legumes, species by species, which could be nodulated by rhizobia that were isolated from other species of legumes in that list but not by rhizobia from other groups of legumes. Although there were clearly some ambiguities, the concept of mutually exclusive inter-nodulation within inoculation groups became an accepted paradigm, and thus the basis of *Rhizobium* taxonomy. As a result, a group of strains shared by an inoculation group

was given species status. The species recognized by this criterion were I. Alfalfa group, R. meliloti; II. Clover group, R. trifolii; III. Pea group, R. leguminosarum; IV. Bean group, R. phaseoli; V. Lupine group, R. lupini; and VI. Soybean group, R. japonicum. A seventh group, the cowpea group (or cowpea miscellany) was not given species status since the legumes that could be cross-inoculated were numerous and taxonomically diverse. There also were apparent subgroups within the cowpea group which did cross-inoculate some subgroups but not others. Fred et al. (1932) considered the cowpea group to be intermediate between the soybean group and the lupine group, because many of the rhizobia from the legume hosts in those groups formed nodules with many of the hosts for the cowpea group.

Rhizobium strains fall naturally into two main divisions on the basis of their physiology and morphology, those which produce rapid growth on rich media and have peritrichous flagella, and those which grow slowly on rich media and have a polar or subpolar flagellum (Jordan and Allen 1974). The fast-growing strains are generally those included in R. leguminosarum, R. phaseoli, R. trifolii, and R. meliloti. The slow-growing strains are usually those included in R. lupini, R. japonicum, and the cowpea miscellany (Jordan and Allen 1974). Several strains from the People's Republic of China nodulate soybean but are similar in growth response and physiology to the fast-growing species of Rhizobium (Keyser et al. 1982). These

strains were described taxonomically as R. japonicum and noted parenthetically to be fast-growing strains (Keyser et al. 1982, Jansen van Rensburg et al. 1983, Heron and Pueppke 1984). This taxonomic problem serves to underscore the overall deficiencies of a taxonomy based on host range. Recently, the taxon R. fredii was proposed for the fast-growing rhizobia that infect soybean (Scholla and Elkan 1984).

In 1964, Graham (1964) revised the taxonomy of the Rhizobiaceae. He recognized previous criticism of the cross-inoculation group concept (Wilson 1944), and based his revision on a numerical taxonomy that compared 100 physiological characteristics. He proposed that R. phaseoli, R. trifolii and R. leguminosarum be consolidated into a single species R. leguminosarum. Agrobacterium tumefaciens and A. radiobacter were to be included in the genus Rhizobium as R. radiobacter. The fast-growing species R. meliloti was retained. Graham also proposed that the slow-growing strains be contained in a newly proposed genus Phytomyxa. The taxonomic revision proposed by Graham was not widely accepted, but some of the general features of his system are included in recently proposed changes. The International Subcommittee on Agrobacterium and Rhizobium recently proposed that the slow-growing strains of Rhizobium be transferred to a new genus Bradyrhizobium gen. nov. (Jordan 1982). This taxon emphasizes the basic physiological difference between the fast-growing and slow-growing strains. Jordan (1984) included most of Graham's

proposed revisions in his recent description of the family Rhizobiaceae. The proposed species are R. meliloti, R. leguminosarum (with the biovars: trifolii, phaseoli, and viceae) and R. loti, which includes strains that nodulate Lotus spp. and related plants. The genus Bradyrhizobium essentially represents the strains for which Graham proposed the name Phytomyxa (Graham 1964). The genus Agrobacterium was retained. This revision addresses many of the deficiencies in the former taxonomic treatments of the Rhizobiaceae. The new taxonomy should greatly facilitate discussions of the comparative genetics of strains of rhizobia, including the genetic basis of symbiotic interaction. However, for this literature review I will continue to use the nomenclature of Jordan and Allen (1974), because all of the literature to be examined follows that system. Nevertheless, the reader should consider the data on the genetics of symbiosis and host range and the comparisons in interaction phenotype in the framework of the biological relationships suggested by the taxonomy described by Jordan (1984).

Genetics of Rhizobium Infection and Nodulation

Much of what is known about the genetics of nodulation has been developed with the four classical species of fast-growing Rhizobium. The three allied species R. leguminosarum, R. trifolii, and R. phaseoli will be discussed together because similar procedures and genetic

probes have been used to study them. Both Ljunggren (1961) and Beringer (1980) cite Krasilnikov (1941) as providing the first evidence for transfer of nodulating ability from strain to strain when he reported the transformation of nonnodulating strains with culture filtrates from nodulating strains. In 1961, Ljunggren reported the transformation of the nonnodulating R. trifolii strain Bart A. The transformed strain, Bart A·2, nodulated clover, had a smooth colony morphology and produced a serological reaction unrelated to that of Bart A and only partially related to the transforming strain. However, one cannot rule out the selection of a contaminant capable of nodulation.

The association of some of the symbiotic functions with plasmids and the development of procedures for genetic manipulation, including recombinant DNA techniques, have greatly increased experimentation on the genetics of nodulation. Plasmids were detected in various strains of several of the Rhizobium spp. (Tshitenge et al. 1975, Nuti et al. 1977). Higashi (1967) reported that R. phaseoli acquired the ablility to nodulate clover with the transfer of an episomal factor from R. trifolii. Zurkowski et al. (1973) used chemical agents to cure strains of R. trifolii of plasmids and reported concomitant loss of ability to nodulate clover. The introduction of the kanamycin-resistance marker of the transposon Tn5 into R. leguminosarum provided a means to both mutate and mark the location of the mutation. This enabled genetic linkage analysis and selection by DNA-DNA homology (Beringer et al.

1978). The presence of the Tn5 marker in the conjugative plasmid, pRL1JI, enabled selection of transconjugants at a high frequency (Johnston et al. 1978). The ability to nodulate peas was restored to plasmid-cured strains of R. leguminosarum by acquisition of pRL1JI. Ability to nodulate peas was transferred with pRL1JI to strains of R. trifolii, a strain of R. phaseoli, and a slow-growing strain of Rhizobium from Cicer (chickpea). These strains retained their ability to nodulate their normal hosts, but the number of nodules per plant was somewhat reduced. In other strains, nodulation ability co-transferred at greater than 95% with bacteriocin production, a natural marker for pRL1JI (Brewin et al. 1980).

R. leguminosarum plasmids with genes for nodulation exist in several incompatibility groups (Brewin et al. 1982). When conjugated into the same cell, the plasmids either formed cointegrates or one of the plasmids was lost. Three sizes of hybrid plasmids were formed after conjugation of pJB5JI, which codes for pea nodulation and nitrogen fixation genes, into strain T37 of R. trifolii. The size of the cointegrate corresponded to a specific Nod and Fix phenotype on pea or clover (Christensen and Schubert 1983). Host-range specifying genes of R. leguminosarum were localized in a 10 kb fragment of pRL1JI by using sequences adjacent to Tn5 insertion-induced nodulation mutants to select cosmid clones with homology (Downie et al. 1983). The insertion of the 10 kb DNA clone enabled a plasmid-cured

R. phaseoli strain to nodulate pea but not Phaseolus vulgaris. The nodules produced on pea were normal appearing and contained typical bacteroids.

At least some of the genes encoding enzymes for nitrogen fixatation are on plasmids in fast-growing species. Nuti et al. (1979) showed that cloned nitrogen fixation (nif) genes from Klebsiella pneumoniae, when used as DNA probes to Southern transfer blots of EcoRI-digested plasmid DNA, hybridized to 1 or 2 unique restriction fragments from several R. leguminosarum strains. Hooykaas et al. (1981) reported that one particular plasmid in R. trifolii encoded both nodulation functions and nif genes. This was demonstrated by conjugating the plasmid, designated "Sym," into Nod^- Fix^- R. leguminosarum, which consequently became Nod^+ Fix^+. The "Sym"-plasmid conjugated into a cured strain of A. tumefaciens enabled it to form nodules on clover but nitrogen fixation did not occur. Similar experiments with "Sym" plasmids from other strains of R. trifolii and from strains of R. leguminosarum yielded very similar results (Hombrecher et al. 1981, Prakash et al. 1981, Hooykaas et al. 1982). The restriction map of "Sym" plasmids from R. leguminosarum, and DNA-DNA homology studies with the "Sym" plasmid from R. trifolii and the Ti plasmid of A. tumefaciens, show considerable conservation of sequences (Prakash et al. 1982b). Some of these conserved regions correspond to areas that are transcribed in bacteroids isolated from nodule tissue, and to regions with homology to nif probes (Prakash et al. 1982a). The use of the term

"Sym" for plasmids which code for some of the functions necessary for symbiosis may serve to confuse the issue of the role of chromosomal genes in symbiosis. The plasmid genes have proven to be the most tractable, so have received the greatest attention. Evidence for the necessity of an appropriate chromosomal background for expression of the plasmid is clear (Beringer 1982), and some genetic evidence for the existence of specific chromosomal genes active in symbiosis is developing (Noel et al. 1984). The actual number of genes which are involved in coding for nodulation specific functions is unknown (Long 1984).

Most of the products of the "symbiotic" genes are unknown. Zurkowski (1980) correlated the presence of the R. trifolii plasmid pWZ2 with the ability of strains to specifically adsorb to clover roots. Cured strains did not bind to the roots but a transconjugant did (Zurkowski and Lorkiewicz 1979). When strains with the plasmid were assayed for binding in the presence of 30 mM 2-deoxyglucose (a hapten of the clover lectin) a reduction of adsorption was observed. Dazzo and Hubbell (1975) described antigenic differences between nodulating and nonnodulating strains of R. trifolii; unfortunately, it is not clear that true sibling strains were used. The possibility of multiple gene differences make it difficult to evaluate the biological significance of the correlation of antigenicity to nodulating ability. Russa et al. (1982) correlated the occurrence of plasmid pUCS202 with differences in

lipopolysaccharides of R. trifolii strains, an observation corroborated by Raleigh and Signer (1982), who selected nodulation-deficient R. phaseoli strains by enrichment of populations for altered surfaces. This was done by screening survivors refractory to infection by phage Fl. These data point to a problem not often addressed in other studies. Raleigh and Signer (1982) found considerable genetic changes in strain physiology in addition to those that had been selected, and it was difficult to identify which of the changes led to the inability to nodulate. Many of the studies reported here are based on the assumption that the only genetic effect of plasmid loss is the alteration of the component under study (i.e., lipopolysaccharide), and the component is correlated only to the effect under study (i.e., nodulation), without careful consideration of what other genetic systems may be disrupted by loss of the plasmid. The plasmid may represent a substantial amount of the potential genetic information of the cell and many of the functions are, as yet, cryptic. Simple correlations with assumptions as to cause and effect in such complex systems with considerable potential for pleiotropic effects are injudicious until specific genes and gene products can be manipulated to test cause and effect unequivocally.

Although much of the discussion has suggested the host-specific nature of the genes encoded on the plasmids of strains of these three species of Rhizobium, some genes are clearly common to all of the strains. Djordjevic et al.

(1983) tested the effect of two self-transmissible "Sym" plasmids, one (pJB5JI) from R. leguminosarum, the other (pBR1AN) from R. trifolii, on the phenotype of various strains of Rhizobium. When conjugated into a cured strain of either R. leguminosarum or R. trifolii, the plasmid conferred the expected Nod^+ Fix^+ phenotype, i.e. nodules formed, on the host of the strain from which the plasmid was derived. But either plasmid could restore the nodulating phenotype on clover to two R. trifolii strains with Tn5-induced "hair-curling" mutations. Neither plasmid restored the wild type to nonmucoid mutant strains of R. trifolii, regardless of whether the mutant was spontaneous or Tn5-induced.

The study of the genetics of R. meliloti has been somewhat slow to develop, but recently it has received more study than the genetics of the other rhizobia. Bechet and Guillaume (1978) provided the first evidence of very high molecular weight plasmids in R. meliloti. These very large plasmids (> 300 megadaltons) were visualized and characterized from several strains (Rosenberg et al. 1981). Rosenberg et al.(1982) extended this observation by demonstrating the presence of the very large plasmid in all of the 27 strains that were examined from diverse origins. Heat treatment-induced deletion mutants that were Nod^- or Fix^- or both and lacked all or part of the very large plasmid (Bånfalvi et al. 1981). The nif genes of R. meliloti were cloned and selected by homology to nif

sequences from K. pneumoniae. These then were used to demonstrate by hybridization analysis that most of the Nod$^-$ mutants lacked at least 24 kb of DNA. The fragments all included several of the nif structural genes as well as some functions essential for nodulation (Bånfalvi et al. 1981, Ditta et al. 1980, Corbin et al. 1982). Transfer of sequences from the very large plasmid of R. meliloti coding for at least some of the nif genes and some nodulation genes into A. tumefaciens or E. coli enabled the recipient bacteria to form nodules or nodule-like structures on alfalfa plants but not on clover (Hirsch et al. 1984, Truchet et al. 1984). Even quite small (< 8.7 kb) fragments were active (Hirsh et al. 1985). When the "Sym" plasmid from R. leguminosarum, which carries genes for uptake hydrogenase activity, was transferred to R. meliloti, the ability of the recipient to form nodules on alfalfa was not impaired, but the uptake hydrogenase activity was expressed very little, if at all (Bedmar et al. 1984).

Transposon mutagenesis has been used to study the genetics of nodulation and nitrogen fixation in R. meliloti. Mead et al. (1982) examined 6000 strains with presumptive Tn5 insertions for auxotrophy and screened the prototrophs on alfalfa plants. They detected 4 Nod$^-$ (0.07%) mutants, 46 Fix$^-$ (0.8%) mutants, and 20 (0.3%) auxotrophs, which suggests that there are either very few genes coding essential functions for nodulation or that Tn5 insertion is not random. Long et al. (1982) described a system for cloning nodulation genes by direct complementation of Nod$^-$

mutants using members of an overlapping cosmid clone bank to map the mutations. Using this system they found that genes essential for nodulation in R. meliloti are located on the very large plasmid within 30 kb of nifK (Long et al. 1982, Zimmerman et al. 1983).

The transfer of RP4 and R68.45 factors into R. meliloti enables the mobilization of the chromosome by conjugation (Kowalczuk and Lorkiewicz 1979). This procedure has allowed the development of linkage maps for chromosomal genes (Kiss et al. 1980, Forrai et al. 1983), which are essentially colinear with those of R. leguminosarum and R. trifolii (Kondorosi and Johnston 1981, Beringer 1980). Of 13 Fix$^-$ mutations mapped, 5 were localized to the chromosome and 8 were extrachromosomal. The chromosomally located Fix$^-$ mutations were not clustered. None of the Nod$^-$ mutants mapped to the chromosome (Forrai et al. 1983). There are several reports of generalized transduction in R. meliloti (Kowalski 1967, Sik et al. 1980, Finan et al. 1984, Martin and Long 1984), but transduction has not been used extensively in genetic studies of this bacterial species, perhaps because of the limitation in size of DNA segments which can be transferred (Beringer 1980).

Very few of the Nod$^-$ mutants have been characterized. Hirsch et al. (1982) reported the infection phenotype of 4 mutants which had previously been derived by Tn5 mutagenesis (Meade et al. 1982). Two of the Nod$^-$ mutants did not induce root hair curling or penetrate host cells. The other two

induced root hair curling, and entered root epidermal cells by some means other than infection thread formation. The mode of entry of the bacteria into epidermal cells was not determined, but it was clear that the process did not maintain cell membrane integrity, because bacteria were observed in cell cytoplasm.

Understanding of the genetics of the slow-growing strains of R. japonicum has lagged behind that for the fast-growing rhizobia, partly because of the truculence of the slow-growing rhizobia to most of the techniques developed for study of the fast-growing rhizobia. Maier and Brill (1976) treated strain 61A76 with nitrosoguanidine and screened 2500 colonies on Corsoy soybean. Five mutants were selected on the basis of lack of nodule development or altered nodule appearance (including reduced leghemoglobin). Two of the five were reported not to form nodules but were competent to fix nitrogen in vitro. Three of the five were Fix^-. It has since been reported that the two strains reported to be Nod^- are actually Nod^+, but nodules are slow to appear (Stacey et al. 1982). Maier and Brill (1978) reported that two strains showed earlier nodulation greater nitrogen fixation, and one of the strains produced more nodules than the parent strain. It is not clear whether these selected strains demonstrated an increase in symbiotic efficiency through simple mutation or whether the changes were partly due to selection for tolerance to the specific growth conditions of the laboratory. Skogen-Hagenson and Atherly (1983) used elevated temperature and either SDS or

ethidium bromide amendment of culture medium, procedures designed to cure plasmids, to produce mutants in strains USDA 74 and 61A76. They reported that none of the strains showed altered plasmid content, but that more than 50 of 133 isolates were symbiotically altered. Of those tested none was reported to be auxotrophic. Forty-four were Nod^- and ninewere Fix^-. The exceptionally high ratios of symbiotic mutants to auxotrophs and of Nod^- to Fix^- make these results unique. If reproducible, the procedure should be very useful for study of R. japonicum genetics.

The role of plasmids in R. japonicum is not well understood. Gross et al. (1979) developed plasmid profiles for a group of "extra-slow-growing" strains indigenous to alkaline soils. Each strain had two to four plasmids, with sizes ranging from 48 to 130 megadaltons. Plasmids of 91 and 118 megadaltons were common to all of the extra-slow-growing strains, but no phenotype was correlated with a particular plasmid. Plasmid content of several slow-growing strains was examined (Haugland and Verma 1981, Masterson et al. 1982). Strains were characterized as usually having one plasmid, but some strains had two or none. When cloned K. pneumoniae nif stuctural genes were used as a probe against R. japonicum plasmid DNA, no hybridization was detected.

The nif genes with homology to cloned K. pneumoniae genes, nifKDH, have been localized in R. japonicum strain USDA 110 (Hennecke 1981, Fuhrmann and Hennecke 1982, Kaluza et al. 1983, Fuhrmann and Hennecke 1983). Strain USDA 110

is a plasmid-less strain. In contrast to the fast-growing strains of Rhizobium, where the nitrogenase structural genes are clustered in one operon (nifKDH), in this strain nifDK represents one operon and nifH is in another operon at least 12 kb away (Kaluza et al. 1983). Hadley et al. (1983) reported that the nifKDH region of K. pneumoniae, when used as a probe on blots of restricted DNA from 17 slow-growing Rhizobium strains, hybridized to at least two EcoRI fragments from each strain, suggesting that the separation between the operons for nif structural genes is common among a number of strains and not unique to USDA 110. Hahn and Hennecke (1984) used a site-directed mutagenesis technique in which Tn5 mutagenesis of the cloned R. japonicum nifDK operon was carried out in E. coli. The operon was transferred by conjugation to R. japonicum by suicide vectors, and stable exconjugants were selected. Mutations within nifD or nifK caused a Nod^+ Fix^- phenotype, whereas Tn5 insertions in the immediate area to either side of nifDK were Nod^+ Fix^+. This is evidence that, unlike R. meliloti, the location of nodulation genes may not be closely linked to nif structural genes in R. japonicum. Hom et al. (1984) reported general mutagenesis of R. japonicum strain USDA 110 with Tn5 carried on a suicide plasmid. Of ten thousand kanamycin resistant mutants, auxotrophs were detected at a frequency of 0.5%. Two hundred mutants were screened on plants and six Fix^- mutants, but no Nod^- mutants, were detected.

These data suggest that the slow-growing strains have a much different genome arrangement than the fast-growing rhizobia. The strains included in R. japonicum have not been shown to have the class of plasmids greater than 300 megadaltons which is nearly ubiquitous in the fast-growing rhizobia. In fact, several strains of R. japonicum seem to have no plasmids at all, and in at least one strain, the genes which are commonly plasmid borne in the fast-growing rhizobia have been mapped to the chromosome. The results of the genetic studies of the fast-growing rhizobia, although providing insight and guidance in the development of suitable experimental approaches for study of R. japonicum genetics, should not be indiscriminantly generalized to the slow-growing strains without critical evaluation of their broader applicability.

Infection and Nodulation of Legumes

The colonization of roots by rhizobia (Roc) is generally believed to be a nonspecific phenomenon. At one time it was assumed that a legume specifically enhanced the growth of nodulating rhizobia, and that such enhancement was one of the underlying causes of the observed specificity of nodulation. The results of experiments measuring root colonization have not yielded clear-cut evidence for such a specific plant effects on colonization. These studies have been reviewed extensively by Fahraeus and Ljunggren (1968) and Vest et al. (1973).

Debate continues as to the role of adsorption of bacteria to roots (Roa) in the specific selection of strains by the plant. The hypothesis that the control of bacterial host range is mediated by the ability of the bacteria to bind to plant surfaces is mostly supported by indirect evidence. Support for this theory is principally in the form of the correlation between the ability of a plant component to bind in vitro to strains of rhizobia and the ability of those strains to nodulate the plant. Such experiments have given rise to the "lectin hypothesis" (reviewed by Dazzo and Hubbell 1982). The hypothesis suggests that plant lectins act as highly selective molecules on or near the surface of roots, where they "recognize" potential microsymbionts by attaching them to reactive sites on the root surface, initiating infection. The ability of the lectin to discriminate between even very closely related rhizobia is thus believed to be the basis of the observed host range of the rhizobia. Correlations of ability of the microsymbiont to bind the host lectin are far from perfect. Even correlations of 5 strains which bind lectin out of 7 strains which nodulate have been cited as validating the lectin hypothesis (Law and Strijdom 1984)! Rarely is an explanation provided for the selective host range of those strains which do not bind lectin in a host-specific manner or those which bind the lectin in a hapten-reversible manner yet do not infect the plant that produces the lectin.

Data are less conclusive in relating bacterial host range to ability to adsorb to roots in experiments where

actual numbers of bound bacteria are determined. Experiments designed to determine the amount of bacterial binding are of three general types: i. measurement of radioactivity of roots to which radiolabled bacteria have been bound, ii. microscopic estimation of the number of bacteria attached to roots, and iii. determination of the number of bound bacteria by grinding root segments, plating the grindate, and extrapolating the number of resulting colonies to the number of bacteria bound to the root segments. The relative advantages and drawbacks of these experimental approaches were reviewed by Pueppke (1984a). A comparison is made in the introduction to Chapter Four of this dissertation of several of those studies as they relate to bacterial host range determination.

The "Hac" phenotype refers to curling of root hairs by infective strains of rhizobia. The curled root hairs are often the ones that contain infection threads in soybean (Ranga Rao and Keister 1978, Turgeon and Bauer 1982, Pueppke 1983). Callaham and Torrey (1981) demonstrated in white clover that most infections arise at the inner curve of strongly curled root hairs, but others occur in nearly straight root hairs. Some have postulated the existence of separate "curling factors," although no such factor is well characterized, and experiments using crude preparations have been ambiguous (Ervin and Hubbell 1985). Hubbell (1981) has proposed a model of root hair curling that explains the curling in terms of asymetric disruption of the elongating

root hair cell wall by bacterial enzymatic degradation, suggesting that curling is an immediate consequence of the incipient infection. This is corroborated in studies of infection of alfalfa by A. tumefaciens containing cloned genes from a region of the R. meliloti "Sym" plasmid shown to have functions essential for nodulation (Hirsch et al. 1984). The strains which had a root hair curling phenotype like the wild-type were those which formed infection threads. The strains which produced various deformations of root hairs, readily discriminated from the wild-type phenotype, did not form infection threads. Sutton et al. (1984) cloned genes from R. japonicum which cause distortion of root hairs of Glycine soja. From the data they present it is impossible to evaluate whether the root hair "curling" is similar to that seen in root hairs with infections or is some other type of distortion, although their photomicrographs suggest the later.

The process of infection thread formation (Inf) was examined in some detail in the small-seeded legumes using techniques of light microscopy which allow direct examination of living plants under nodulating conditions (Fahraeus and Ljunggren 1968, Ljunggren 1969, Li and Hubbell 1969, Callaham and Torrey 1981). In those plants infection thread formation is highly specific; strains which are capable of nodulating a plant are found to form infection threads; conversely, those strains which can form infection threads, with rare exceptions, produce nodules.

The formation of infection threads in soybean was first described by Bieberdorf in 1938. He noted that infection leading to nodulation generally was by means of infection threads in root hairs, though he stated that infection directly through root epidermis occurred on occasion. Direct penetration of soybean root epidermis has not been corroborated, though infection by direct penetration or through natural wounds leads to nodulation of some tropical legumes (Allen and Allen 1940, Ranga Rao 1977, Chandler 1978, Chandler et al. 1982). Infection threads have been described in soybean by Ranga Rao and Keister (1978), Newcomb et al. (1979), Turgeon and Bauer (1982), Pueppke (1983), and Heron and Pueppke (1984). Turgeon and Bauer (In press) described root hair infection of soybean at the ultrastructural level. Pueppke (1983) demonstrated that when eight lines of soybean, four lines of wild soybean, and one cowpea cultivar were inoculated separately with 18 Rhizobium strains, infection threads were formed in all combinations which also formed nodules but were not formed in any nonnodulating combination. The infection threads almost exclusively were formed in root hairs which were distal to the region with mature root hairs at the time of infection.

The formation of nodules, the Nod phenotype, though not an early infection event, is often used as a quick and easily observed assay for mutations affecting infection. Although this is understandable, particularly in large screening experiments, too often the presence of nodules is

assumed, prima facie, to be evidence that all of a set of specific infection events haveoccurred. Hirsch et al. (1982, 1984) have demonstrated that nodules or "pseudonodules" are produced on alfalfa by mutant strains of R. meliloti which have altered infection phenotypes, including Hac$^-$ and Inf$^-$. In many studies the reason for the relatively small number of Nod$^-$ mutants compared to Fix$^-$ mutants may be that some mutants had altered infection phenotypes but still produced nodules. Most or all of the steps described above, including additional phenotypically defined steps such as release of the bacteria from the infection thread, are essential in legume-microsymbiont combinations with only the root hair infection mechanism. Symbiotically deficient nodules that are superficially normal may form even though all of the steps do not occur. It is not yet clear which of the infection steps are necessary for the production of a macroscopic nodule.

An additional problem with using nodulation as a screen for infection events is the assumption that infection proceeds only by means of infection threads in root hairs. For several tropical legumes nodulated by slow-growing rhizobia this is not the case (Allen and Allen 1940, Ranga Rao 1977, Chandler 1978, Chandler et al. 1982). In an uncorroborated report Bieberdorf (1938) suggested that soybean could likewise be infected by direct penetration of the root epidermis in addition to infection threads in root hairs. If two pathways were to exist for infection and they

depended on separate sets of genes in the bacterium, screening for mutation in infection by nodule production would cause many of the mutations to be missed due to nodulation by the remaining pathway even when one pathway was blocked.

Phenotypic Nodulation Response of Soybean

Variation in nodulation phenotypes of soybean cultivars was noted early in the century by Vorhees (1915), who reported that in plots which had been inoculated with either of two commercial soybean inocula, the soybean variety Haberlandt had no nodules while five other varieties were well nodulated. Haberlandt had no nodules, even in plots where it was interplanted with a cultivar which was well nodulated. Vorhees concluded that different varieties of soybean carried different levels of resistance to association with symbiotic bacteria. In an addendum to Vorhees' report, Morse (1915) notes that in subsequent years he observed efficient nodulation of the variety Mammoth in plots that did not support nodulation of the varieties Acme and Tokio. Morse commented that in tests other than that reported by Vorhees, Haberlandt nodulated as well as other varieties, suggesting that Vorhees' data were indeed best explained on the basis of strain-specific resistance rather than basic incompatibility of the cultivar to nodulation. In addition, Briscoe and Andrews (1938) observed that differences between the nodulation responses of varieties of soybeans were as great as the differences between cowpeas

and soybeans when reciprocal tests of their rhizobia were made.

In a study covering three consecutive years (Caldwell and Vest 1968, Vest et al. 1973), bacteria from nodules on 17 soybean genotypes were serotyped. The cultivar Lee was used as a check variety. The population of strains nodulating the cultivar Pickett was not significantly different from the backcross parent Lee, but many of the soybean lines less closely related were significantly different when the serogroups of strains which formed nodules were compared. This was true even for varieties grown side-by-side. The cultivar Peking was included in this study. Even though it was planted in a field known to have a natural population of mixed strains of R. japonicum of which strain 110 was a major component, fewer than 1% of the nodules on Peking contained strain 110. And yet, when Peking was inoculated with strain 110 in pots of sterile soil, the plants were nodulated very efficiently. Of course, competition between the rhizobia may account for some of these differences.

Nodulation Restrictive Soybean Genotypes

In 1954, Williams and Lynch reported the inheritance of a nonnodulating character in soybean. The trait was found in 1947 among breeding selections from a cross between Lincoln and a line selected from a Lincoln X Richland cross. Resistance to nodulation segregated as a single recessive character, with the homozygous recessive plant expressing

the resistance to nodulation. Selfs of both parents and test crosses between the parents all gave the normal nodulating phenotype, indicating that the characteristic apparently had arisen as a mutant. Williams and Lynch (1954) called the gene no and its dominant allele No. Caldwell (1966) renamed these genes rj_1 and Rj_1 to conform with general soybean genetic nomenclature.

Genotypes conditioning resistance to Rhizobium infection have been characterized in several legumes, other than soybean. The best characterized phenotypic expression of such a genotype is in red clover (Nutman 1949). Resistance is expressed as lack of any infection threads, although some root hair deformation occurs upon inoculation with R. trifolii. The resistant genotype is described as homozygous recessive, rr. A cytoplasmic factor, p, interacts with rr and is inherited in a complex manner. A genotype resulting in a Fix^- phenotype is designated ii. The gene i segregates independently of r (Nutman 1949).

Two independently segregating genes affect symbiosis in field peas. The genotype sym_2sym_2 conditions resistance to nodulation, and sym_3sym_3 prevents nitrogen fixation (Holl 1975). A Fix^- phenotype is produced in crimson clover (T. incarnatum), irrespective of strain, by the single recessive gene pair rt_1rt_1, with possible modifiers (Smith and Knight 1983). The genetic constitution of phenotypically Nod^- peanut (Arachis hypogaea) requires the independently segregating double recessive gene complement, $n_1n_1n_2n_2$

(Nigam et al. 1980). None of these nodulation resistant genotypes has been characterized at the cellular or biochemical level. It is interesting to note that even peanut, which is infected through natural wounds (Allen and Allen 1940, Chandler 1978), often considered a passive mode of infection, expresses resistance in the recessive genotype.

Several soybean genes condition strain-specific Fix^- phenotypes. In each case the Fix^- phenotype is conditioned by the dominant allele. The $\underline{Rj_2}$ allele conditions against strains of the 122 and cl serogroups (Caldwell 1966), the $\underline{Rj_3}$ allele conditions against strain USDA 33 (Vest 1970), and the $\underline{Rj_4}$ allele conditions against strain USDA 61 (Ham et al. 1971). One or both of the alleles $\underline{Rj_2}$ or $\underline{Rj_4}$ are present in 30% of the plant introduction lines, but have generally been selected against in breeding programs and are present in only a few named cultivars (Devine and Breithaupt 1980c, 1981, Devine 1984a). When plants carrying the dominant allele are inoculated with the restricted strains, they produce small nodules or nodule-like proliferations on the roots. Pueppke (1983) showed that the cultivar Hardee, which carries the genotype $\underline{Rj_2Rj_3}$, developed infection threads which appeared to be normal with strain USDA 138 (cl serogroup), so the block in nodule function appears to occur late in development. Although $\underline{Rj_2}$, $\underline{Rj_3}$, and $\underline{Rj_4}$ are often described with $\underline{rj_1}$ as a group of "nodulation restrictive genes," clear differences are apparent. Whereas the $\underline{rj_1rj_1}$ genotype conditions against most strains of *R. japonicum*

with no nodules or nodule-like structures formed by restricted strains; the other "nodulation restrictive genes" are restrictive only to a few strains or specific serotypes, and nodules or nodule-like proliferations are formed. $\underline{Rj}_2$, $\underline{Rj}_3$, and $\underline{Rj}_4$ restrict symbiotic effectiveness not nodulation so the term "nodulation restrictive genes" is a misnomer for them.

The $\underline{rj}_1\underline{rj}_1$-soybean was originally described as nonnodulating (Williams and Lynch 1954). Clark (1957) found that a few nodules were formed by a few strains when plants were grown hydroponically using sand as the support medium, but the plants were incapable of being nodulated in soil. The typical nodulation response in sand culture was about one nodule per plant. Nodulation response was reduced for plants grown in sand amended with soil, and no nodules were formed when the sand was amended with 10% bentonite clay.

Isolines of soybean differing at the $\underline{Rj}_1$ locus were found in one study of root colonization to harbor approximately equal numbers of rhizobia (Clark 1957). Elkan (1962) later reported larger numbers of rhizobia in rhizospheres of $\underline{rj}_1\underline{rj}_1$-soybean than in its isoline for approximately the first 40 d of plant growth in the field. Clark (1957) reported no differences in the kinds or amounts of amino acids in the two isolines, but Hubbell and Elkan (1967b) noted that roots of uninoculated $\underline{Rj}_1$-plants contained larger amounts of protein and reducing sugars and smaller amounts of free amino acids than did uninoculated

$\underline{rj_1}\underline{rj_1}$-plants. The biological significance of this latter finding is not apparent in light of Elkan's (1962) earlier data on root colonization, which certainly suggests no basic growth inhibition of rhizobia. Elkan (1961) suggested that the $\underline{rj_1}\underline{rj_1}$-soybean produced a nodulation-inhibiting excretion capable of a highly significant reduction of nodulation on the $\underline{Rj_1}$-genotype. The amount of nitrate added to culture medium for container-grown plants, however, was sufficient to have a potential effect on nodule number. Eskew and Schrader (1977) reexamined the putative nodulation-inhibiting excretion from $\underline{rj_1}$-soybean using modifications of Elkan`s (1961) experimental design. They found no statistically significant reduction in nodule number due to co-cultivation of nodulating plants with $\underline{rj_1}$-isolines, but a strong inhibitory effect of nitrate was noted.

Hubbell and Elkan (1967a) compared thephysiological characteristics of strains of R. japonicum with differential abilities to nodulate isogenic lines of soybean differing at the $\underline{Rj_1}$ locus. High measurable indoleacetic acid formation, low indoleacetic acid destruction, formation of large amounts of capsular material, failure to metabolize nitrate, and failure to reduce triphenyl tetrazolium chloride were properties associated with ability to nodulate both normal and mutant soybean. Stains with the opposite properties generally were able to nodulate only the normal soybean. A mode of infection could not be educed from correlations

between physiological characteristics and nodulation phenotype.

Devine and Weber (1977) observed that many R. japonicum strains capable of overcoming rj_1-conditioned resistance to nodulation produced a previously reported soybean foliar chlorosis (Erdman et al. 1956, Johnson and Means 1960). The chlorosis symptoms were ascribed to the formation of rhizobitoxine, 2-amino-3-hydroxypropoxyvinylglycine by the bacteria (Owens and Wright 1965, Owens 1969, Giovanelli et al. 1971, Owens et al. 1972). Devine and Weber (1977) suggested that the production of rhizobitoxine might enable the infection of rj_1rj_1-soybean by overcoming strains. This was examined indirectly by Devine and Breithaupt (1980b), who tested the effect of three temperature regimes on nodulation and chlorosis. The effects were opposite, in that the chlorosis symptoms were greatest at the highest temperature (32 C), but the most nodules were formed at the low and intermediate temperatures (21 C and 27 C). No evidence was found for a diffusible compound capable of endowing the rj_1-incompatible strains with ability to nodulate rj_1rj_1-soybean (Devine et al. 1981). The ethoxy analog of rhizobitoxine, when added to bacteria and used to inoculate soybeans in Leonard jars, did not modify the nodulating ability of strains of R. japonicum on rj_1rj_1-soybean (Devine and Breithaupt 1980a). Devine (1984a) concludes from these data that rhizobitoxine probably has no enabling role in infection. Devine suggests that the rhizobitoxine is only correlated with the rj_1rj_1-overcoming

strains due to fixation of the separate genetic factors in the same population by "random drift."

Devine et al. (1980) determined that the $\underline{rj_1}\underline{rj_1}$-resistance was not a basic incompatiblity with the nonnodulating stains. When the strains capable of nodulation and those which were not were mixed and used as inoculum, 32% of the resulting nodules on $\underline{rj_1}\underline{rj_1}$-soybean contained both strains, 36% contained only the usually nonnodulating strain, and 32% contained only the usually nodulating stain.

The mode of infection of $\underline{rj_1}\underline{rj_1}$-soybean has not been determined. Nutman (1981) notes that the $\underline{rr}$ phenotype of red clover conditions inability of the plant to form infection threads; and, since the soybean resistance to nodulation is likewise a recessive trait, it seems likely to condition a similar block early in infection. Devine (1984a) notes that no nodules or nodule-like proliferations are formed on roots inoculated with incompatible strains, and likewise suggests that the block is early in infection. Tanner and Anderson (1963) examined soybean roots for infection in root hairs but unfortunately were unable to find infection threads in either the $\underline{rj_1}\underline{rj_1}$-soybean or the normally nodulating line.

Perspective

The challenge remains to find the point at which the $\underline{rj_1}$-plant blocks infection and to elucidate the pathway of

infection for those strains which can overcome the $\underline{rj_1}\underline{rj_1}$-resistance to nodulation. These problems are the focus for the studies reported in this dissertation. The function of temperature on nodulation number and pattern was studied to find the conditions under which infections were most likely to be observed in the $\underline{rj_1}\underline{rj_1}$-soybean. The hypothesis that bacterial adsorption has a role in determining differential nodulation ablilty of strains between normally nodulating and restrictive lines of soybean was tested. The plasmid content of strains was determined and attempts were made to alter the genetic complement of strains, in search of clues to the genetic basis for infection. Finally, roots were examined using light and scanning electron microscopy to determine the phenotype of infection at the cellular level.

CHAPTER THREE
EFFECT OF TEMPERATURE ON NODULATION OF SOYBEAN ISOLINES DIFFERING AT THE $\underline{Rj_1}$ LOCUS

Introduction

The nodulation restrictive genotype of soybean, $\underline{rj_1rj_1}$, identified as a spontaneous mutant in a soybean breeding program, was reported by Williams and Lynch in 1954. Initial work determined that one genetic locus is involved in conditioning the restrictive phenotype and that the homozygous recessive genotype is required for expression of the trait. The alleles originally were named no and NO for nonnodulating, but since have been redesignated $\underline{rj_1}$ and $\underline{Rj_1}$ to conform to currently accepted terminology for soybean genetics (Caldwell 1966).

It once was believed that the nodulation restrictive plants are unable to be nodulated (Williams and Lynch 1954, Caldwell 1966). Now it is clear that although most strains of Rhizobium japonicum are unable to nodulate these plants, several strains produce a small number of nodules on plants grown in hydroponic culture (Clark 1957). These strains are called the "overcoming" strains because they overcome the plant resistance. Devine and Breithaupt (1980b) reported a temperature effect on nodulation of the soybean cultivar Clark and its nodulation-restrictive isoline Clark-$\underline{rj_1}$ by

two overcoming strains. The trends in nodulation response of both isolines to temperature are similar. The two bacterial strains have different temperature optima for nodulation with the greatest number of nodules per plant formed at 27 C and 21 C, respectively, for the two strains. Fewer nodules were formed at 32 C for both strains (Devine and Breithaupt 1980b).

Bhuvaneswari and colleagues (Bhuvaneswari 1981, Bhuvaneswari et al. 1980, 1981) developed a model for nodulation of soybean. The model predicts that most nodules will be clustered near the point that represents the position of the root tip at the time of inoculation. The model is supported by a correlation between nodule distribution and the position of areas that had immature root hairs or had not yet developed root hairs at the time of inoculation. This developmental model of nodulation is extended by the observations of Pueppke (1983) and Calvert et al. (1984), who demonstrated that the formation of infection threads is the developmentally restricted event in soybean and two other legumes. In accordance with this model, no nodules are expected to form above the zone of developing root hairs on the primary root (Bhuvaneswari et al. 1980).

The objectives of my study were to *i*. find the temperature optima for overcoming strains, *ii*. test the appropriateness to *rj*$_1$*rj*$_1$-soybean of the Bhuvaneswari model of transient susceptibility of root cells to infection leading to nodulation, and *iii*. extend the study (Devine and

Breithaupt 1980b) of the effect of temperature on nodulation of Clark and Clark-rj_1 isolines to additional overcoming strains.

Materials and Methods

The bacteria all were obtained from the U. S. Department of Agriculture, Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville, MD, courtesy of H. H. Keyser, D. F. Weber, and R. Griffin. All bacteria are USDA strains of *R. japonicum*. The overcoming strains used were 61, 84, 94, and 119. The nonovercoming strain 110 was used as a control in all experiments. The bacteria were maintained at 4 C on yeast extract-mannitol agar slants (Vincent 1970).

Seeds of *Glycine max* (L.) Merr. cultivar Clark-L1 (Rj_1Rj_1) and the nodulation restrictive isoline of Clark-L1, L63-1889 (rj_1rj_1), were obtained from R. L. Bernard, USDA Regional Soybean Laboratory, University of Illinois, Urbana, and D. A. Phillips, Agronomy and Range Science Department, University of California, Davis. The isolines are designated Clark and Clark-rj_1 according to the nomenclature of Devine and Breithaupt (1980b). Seeds were surface disinfested by soaking in 50% ethanol for 2 min with agitation, rinsing in deionized water, and then shaking in 0.5% aqueous sodium hypochlorite for 2 min. Seeds were washed for 20 min in running deionized water and were germinated in the dark on water agar plates for 4 to 5 d at

22 C, 27 C, or 32 C, depending on the temperature to be used for nodulation experiments.

Inoculum was produced from 50 ml log-phase cultures grown in liquid gluconate-mannitol medium (Bhuvaneswari et al. 1977) at 28 C with rotory shaking at 120 rpm. Bacterial suspensions were centrifuged at 7500 x g for 10 min and the bacteria were resuspended in sterile nitrogen-free Jensen's plant-growth solution (Vincent 1970). Cell concentration was adjusted turbidimetrically to 5 x 10^8 cells/ml. Seedlings with roots approximately 4 cm long were inoculated by dipping the roots for 10 min in the bacterial suspension. Inoculated seedlings were placed, 2 per pouch, in autoclaved plastic growth pouches (Northrup King Seed Co, Minneapolis, MN) containing 15 ml of Jensen's solution. The surface of the growth pouch was marked at the location of the primary root tip of each plant (Bhuvaneswari et al. 1980b). This mark was designated the root tip mark (RTM).

Plants were grown for 30 d at continuous temperatures of 22, 27, or 32 C with a 12 hr light/dark cycle in a Conviron E-15 growth chamber with 900 uE/m^2/sec (400-700 nm) irradiance at canopy height. Each temperature experiment was repeated 3 times with 6 plants of Clark and 10 plants of Clark-rj_1 tested for each R. japonicum strain in each experiment. Appropriate control plants sham-inoculated with sterile Jensen's solution were included in each experiment. Plants were watered as needed with deionized water.

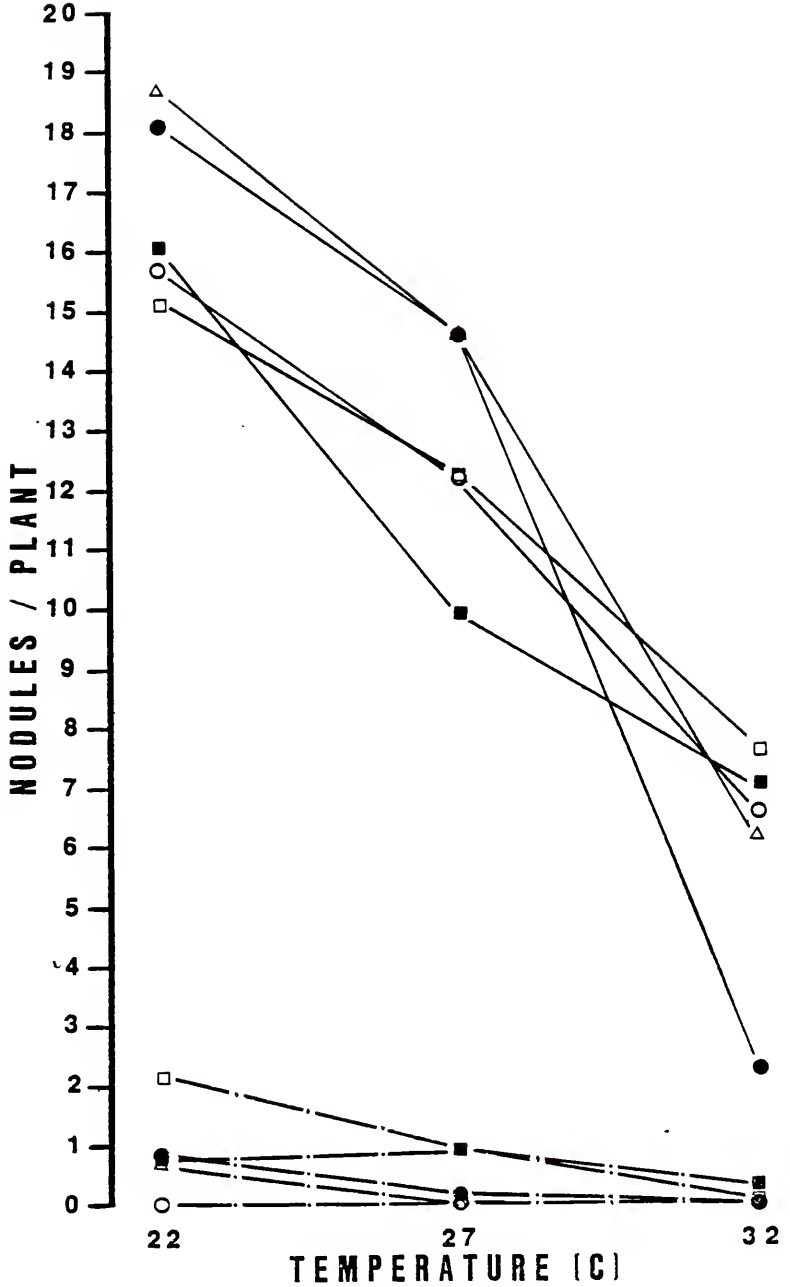
The distance (to the nearest mm) from the RTM to the root crown and from the RTM to each nodule on the primary

root was measured on each plant at harvest. The number of nodules on secondary roots was counted. Data were analyzed by analysis of variance using the general linear models (GLM) procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Computing was done utilizing the facilities of the Northeast Regional Data Center of the State University System of Florida.

Results

The mean number of nodules per plant on Clark ranged from 2 to 8 at 32 C, up to 15 to 19 at 22 C (Figure 3.1, Table 3.1). On Clark-*rj*$_1$ inoculated with overcoming strains, the mean number of nodules ranged from 0 to about 0.2 nodules per plant at 32 C, to from 0.7 to 2.2 nodules per plant at 22 C (Table 3.2, Figure 3.1). Nodules were not formed on Clark-*rj*$_1$ inoculated with the nonovercoming strain 110 or on plants sham-inoculated with plant-growth medium. Analysis of variance was conducted using the number of nodules per plant as the dependent variable. In a preliminary test, the responses of Clark and Clark-*rj*$_1$ were demonstrated to be significantly different. All subsequent analysis thus was conducted separately for the two genotypes to avoid heterogeneity of variance. Temperature significantly ($p < 0.01$) influenced nodule number for each plant genotype. F-values for strain, replication, and the interactions of each of the independent variables were

Figure 3.1. The mean number of nodules formed per plant at three temperatures with five strains of *Rhizobium japonicum*. Plants were dip inoculated in suspensions (5×10^8 cells/ml) of one of the strains indicated, placed in plastic growth pouches, and grown at constant temperature for 30 d. For each strain at each temperature the experiment was replicated three times with six plants per treatment for Clark and ten plants per treatment for Clark-rj_1. Treatments with Clark are indicated by the solid line, Clark-rj_1 with the broken line. □ = strain 61, ■ = strain 84, ● = strain 94, ○ = strain 110, and △ = strain 119.


NODULES / PLANT
20
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2
1
0
22
27
32
TEMPERATURE (C)

Table 3.1. The effect of temperature on the nodulation of Clark soybean by *Rhizobium japonicum*

Strain	Temperature 22 C	27 C	32 C
61	15 ± 6[a]	12 ± 4	8 ± 3
84	16 ± 7	10 ± 3	7 ± 3
94	18 ± 5	15 ± 6	2 ± 2
110	16 ± 9	12 ± 5	7 ± 3
119	19 ± 7	15 ± 6	6 ± 3

[a] Mean number of nodules per plant for 3 replications with 6 plants per replication ± standard deviation.

Table 3.2. Effect of temperature on the nodulation of Clark-rj_1 *Rhizobium japonicum*

Strain[a]	Temperature		
	22 C	27 C	32 C
61	2.2 ± 2.0[b]	1.0 ± 1.2	0.1 ± 0.3
84	0.8 ± 1.4	0.9 ± 1.7	0.2 ± 0.7
94	0.9 ± 1.4	0.1 ± 0.4	0
110	0	0	0
119	0.7 ± 1.1	0	0

[a] Strain 110 is a nonovercoming control. All others are overcoming strains.

[b] Mean number per plant for 3 replications with 10 plants per replication ± standard deviation.

not significant. A dramatic indication of the effect of temperature on nodulation of Clark-*rj*$_1$ is provided when the data are expressed as the percentage of plants developing at least one nodule per plant after inoculation with an overcoming strain (Table 3.3). At each temperature a total of 120 plants was inoculated with one of the four overcoming strains; of those, 4% of the plants were nodulated at 32 C, 23% at 27 C and 44% at 22 C.

The ratio of primary to secondary nodules ranged from 1:0.6 to 1:0.9 on Clark. On Clark-*rj*$_1$ the ratios were 1:9 at both 32 C and 27 C, and 1:4 at 22 C.

Histograms were developed for each interaction of bacterial strain x isoline for each temperature as described for various other legume x microsymbiont combinations (Bhuvaneswari 1981, Bhuvaneswari et al. 1980, 1981, Halverson and Stacey 1984, Heron and Pueppke 1984). These are shown in Figures 3.2 and 3.3. In most interactions with overcoming strains, nodules formed well above what has been considered the zone of infectibility, defined as that area which has only emerging root hairs or no root hairs at the time of inoculation. This type of anomolous nodulation is seen at 22 C and 27 C for combinations of Clark with the strains 61, 84, and 94, each an overcoming strain. A pattern of nodulation similar to those described as fitting the nodulation model of Bhuvaneswari (1981) is seen in the combination of Clark with strain 110, a nonovercoming strain, at 22 C and 27 C. From the nodule profiles

(Figure 3.2), it can be seen that the pattern of nodulation at 32 C of Clark by all of the tested bacterial strains produced flattened peak and a population of nodules displaced downward with respect to the profiles observed at 22 C and 27 C. This downward displacement is clearly evident when the data are expressed as the mean distance of all primary root nodules from the RTM (Table 3.4). The nodule profiles of the overcoming strains on Clark-$\underline{rj}_1$ showed sparse nodulation down the length of the root from just above the RTM.

Discussion

The soybean cultivar Clark and its isoline, Clark-$\underline{rj}_1$, were used by Devine and Breithaupt (1980b) to study the effect of temperature on nodulation. They tested the two overcoming strains USDA 61 (used in this study) and 76. Strain 76 produced the most nodules on Clark-$\underline{rj}_1$ at 27 C with few nodules formed at 21 C or 32 C. The combination of Clark-$\underline{rj}_1$ with strain 61 developed the most nodules at 21 C (7.5), with 6.4 and 3.1 nodules at 27 C and 32 C, respectively. In my study the slope of the regression of a plot--number of nodules versus temperature--was similar to that observed by Devine and Breithaupt (1980b), but the absolute numbers of nodules per plant were lower (Figure 3.1). All of the overcoming strains that I tested responded similarly to strain 61 in the previous study, except strain 84 which had slightly fewer nodules at 22 C than at 27 C.

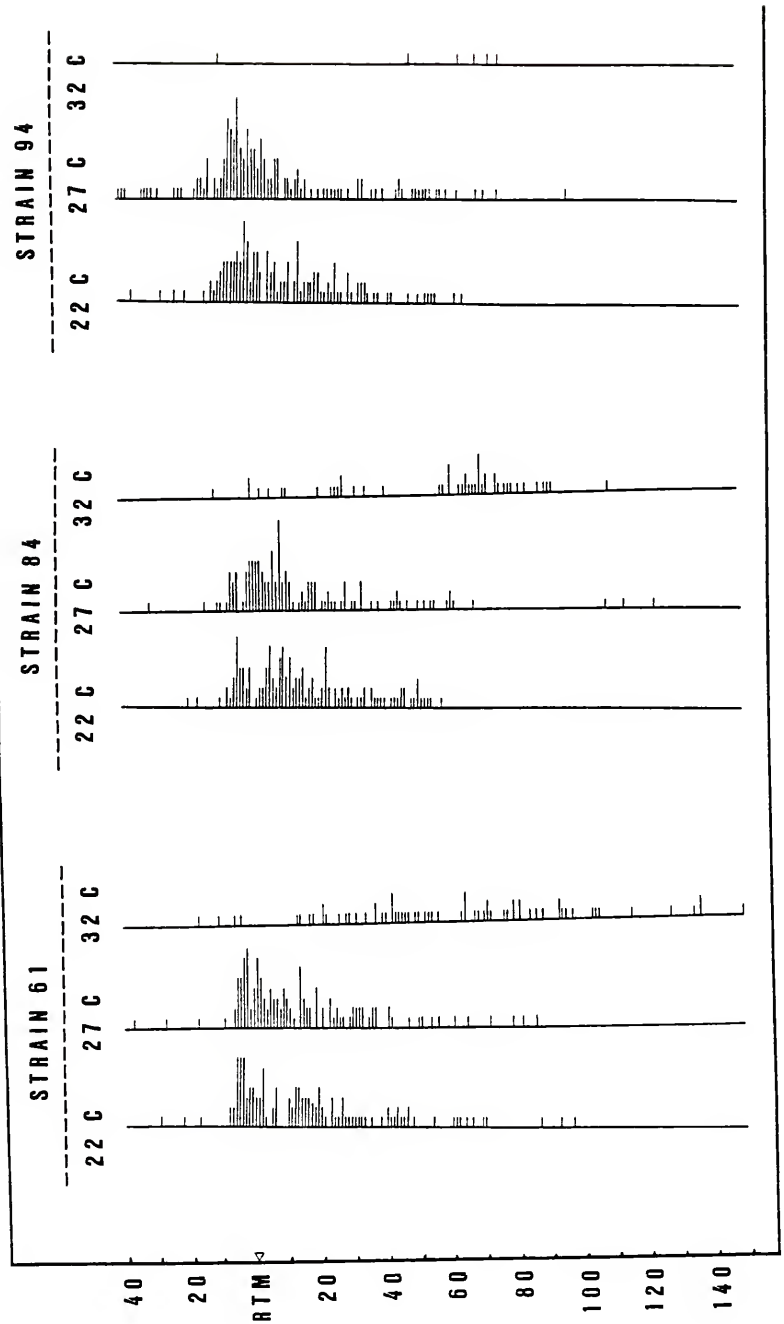
Table 3.3. The percentage of Clark-rj_1 soybean plants nodulated by overcoming strains of *Rhizobium japonicum* at three temperatures

	Temperature								
	22 C			27 C			32 C		
Strain	pri	sec	any[a]	pri	sec	any	pri	sec	any
61	23	73	77	10	50	53	3	3	7
84	3	30	30	7	30	30	0	10	10
94	13	27	40	0	10	10	0	0	0
119	3	27	30	0	0	0	0	0	0
Total[b]	11	39	44	4	23	23	1	3	4

[a] Percentage of 30 plants nodulated (3 replications x 10 plants) at the locations indicated. Pri = percentage of plants with at least one nodule on the primary root. Sec = percentage of plants with at least one nodule on the secondary roots. Any = percentage of plants with at least one nodule.

[b] Percentage of all plants in each temperature experiment with at least one nodule (120 plants per temperature).

Figure 3.2. Frequency histogram of nodule distribution on the primary root of Clark soybean. Plants were grown 30 d in plastic growth pouches at 22, 27, or 32 C after dip inoculation with one of the five strains of Rhizobium japonicum indicated. Each nodule on the primary root was measured to the nearest millimeter from the RTM (root tip mark, placed on the growth pouch at the time of inoculation). The frequency diagrams represent the plants from three replications at each temperature with six plants per treatment.


STRAIN 61
22 C
27 C
32 C
STRAIN 84
22 C
27 C
32 C
STRAIN 94
22 C
27 C
32 C
RTM
40
20
20
40
60
80
100
120
140

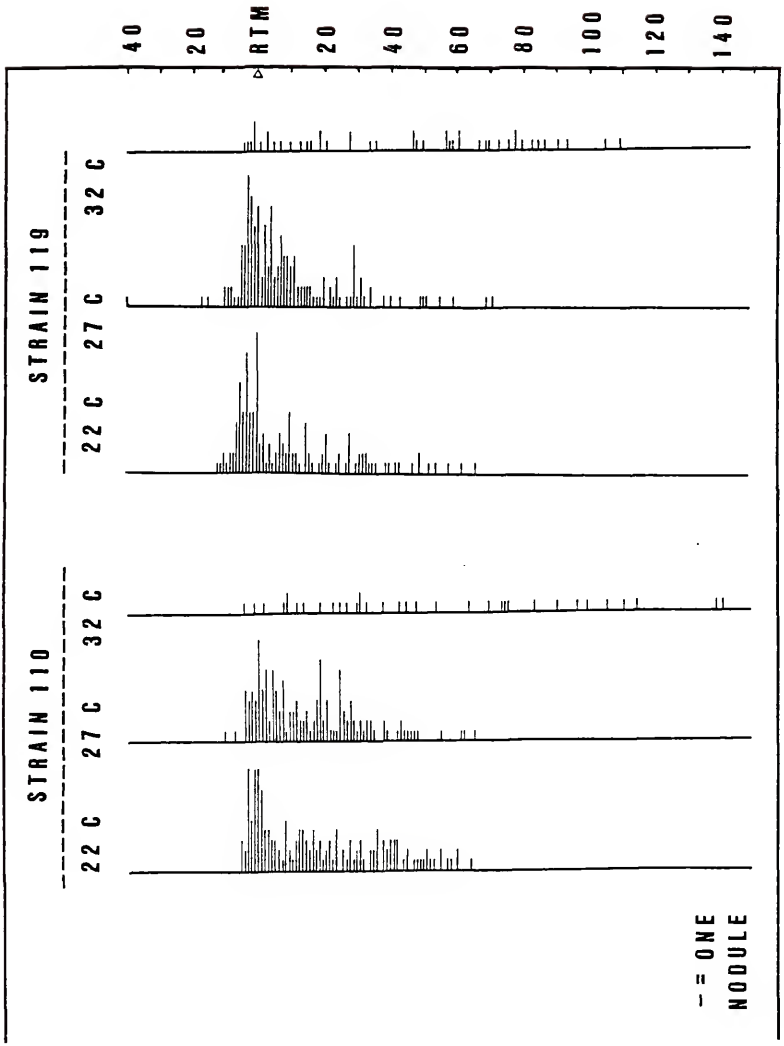


Figure 3.2 Continued

Figure 3.3. Frequency histogram of nodule distribution on the primary root of Clark-*rj*$_1$ soybean. Plants were grown 30 d in plastic growth pouches at 22, 27, or 32 C after dip inoculation with one of the four strains of *Rhizobium japonicum* indicated, or the control strain 110. Each nodule on the primary root was measured to the nearest millimeter from the RTM (root tip mark, placed on the growth pouch at the time of inoculation). The frequency diagrams represent the plants from three replications at each temperature with ten plants per treatment. No nodules were observed on plants inoculated with strain 110 (not shown) at any temperature.

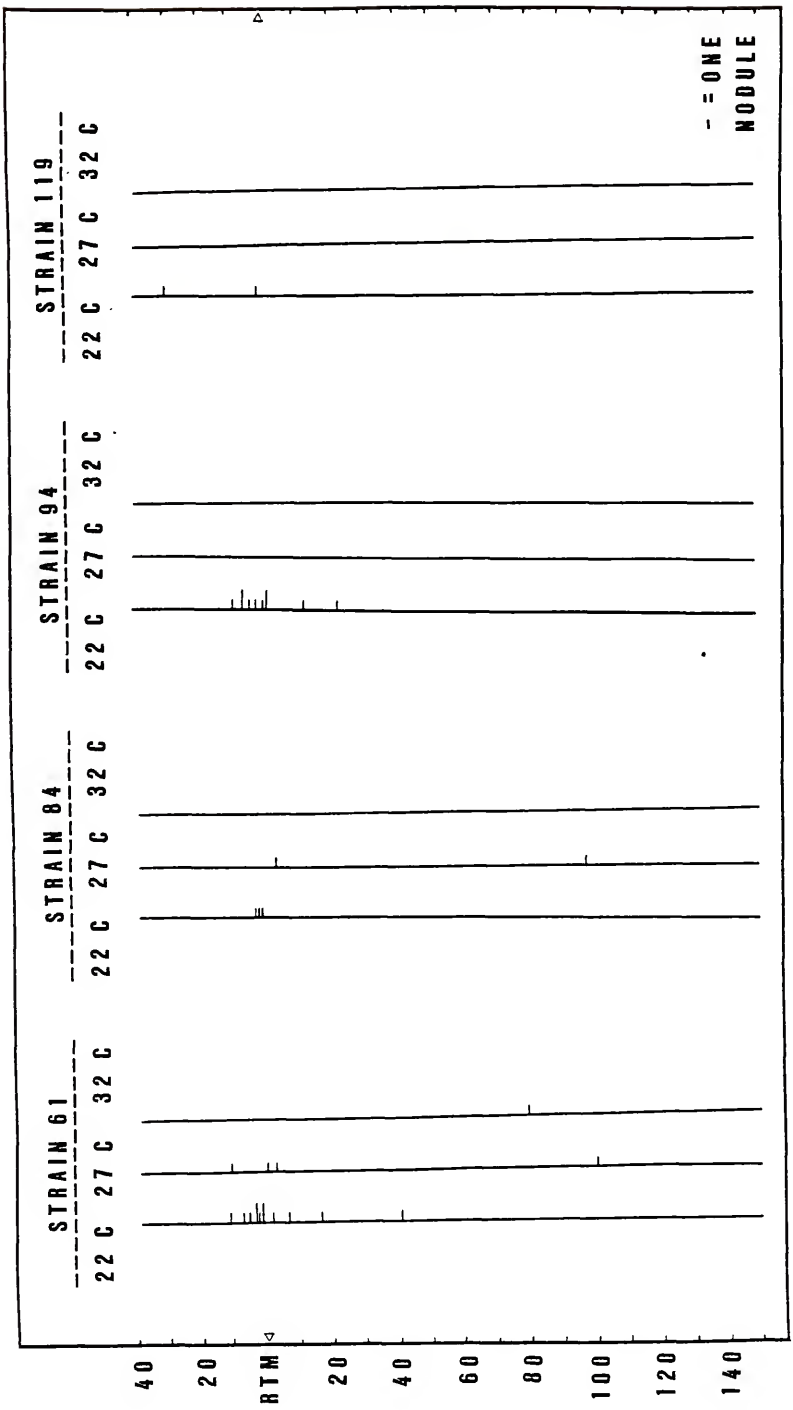

STRAIN 61
22 C 27 C 32 C
STRAIN 84
22 C 27 C 32 C
STRAIN 94
22 C 27 C 32 C
STRAIN 119
22 C 27 C 32 C
- = ONE NODULE
RTM
40
20
20
40
60
80
100
120
140

Table 3.4. Mean distance of primary root nodules on Clark soybean from the root tip mark at time of inoculation

Strain	Temperature		
	22 C	27 C	32 C
61	16 ± 2[a]	13 ± 2	61 ± 5
84	16 ± 2	16 ± 2	56 ± 4
94	11 ± 2	7 ± 2	51[b]
110	16 ± 1	14 ± 1	53 ± 7
119	9 ± 1	9 ± 1	43 ± 5

[a] Mean nodule distance in millimeters for 18 plants (3 replications x 6 plants) ± standard error of the mean. Measurements for nodules above the RTM were given a negative value.

[b] Only one nodule produced on the primary root of a plant in 3 replications of this treatment.

At 22 C only the combination of the nonovercoming strain 110 and Clark-$\underline{rj}_1$ failed to produce nodules on at least some of the plants. At 27 C, Clark-$\underline{rj}_1$ failed to form nodules with strain 119 as well as with strain 110, and at 32 C no nodules were formed by strains 94 and 119, as well as 110. The effect of temperature on nodulation of both Clark and Clark-$\underline{rj}_1$ had the same trend (Figure 3.1), although the number of nodules per plant was much different for the two plant types. Nodulation of nonovercoming strain 110 on Clark was affected by temperature in a manner similar to the overcoming strains, but strain 110 did not nodulate the Clark-$\underline{rj}_1$ isoline at any temperature.

Reports have been made on the effect of temperature on numbers of soybean nodules per plant both in the greenhouse and field, as well as under controlled conditions (Devine and Breithaupt 1980b, Munevar and Wollum 1982, Weber and Miller 1972), but this study is the first to examine the effect of temperature on the pattern of nodulation. The pattern of nodulation of strain 110 on Clark soybean at 27 C and 22 C was similar to the pattern reported by others for compatible interactions and follows that predicted by the model of transient susceptiblity to nodulation (Bhuvaneswari 1981, Bhuvaneswari et al. 1980, 1981). The general shape of the profiles for the overcoming strains on Clark is similar to strain 110 on Clark, suggesting that at least most of the nodules that arise are the result of infections constrained by developmental processes to areas of susceptibility, as

defined by the region of the root on which the root hairs are immature or are not yet formed at the time of inoculation. Overcoming strains 61, 84, and 94 produced some nodules well above that region known to be infectible by the model of infection. The presence of these nodules can be explained in two ways. First, immature root hairs may exist in this region and remain infectible after the surrounding root hairs have matured. Alternatively, these overcoming strains may have an additional infection sequence that is not restricted to areas with developing root hairs. If the first is true, one would expect that strain 110 and other nonovercoming strains also would produce nodules in this area, at least occasionally. Since nodules are produced in this area only by overcoming strains, an alternative infection mechanism is suggested.

The pattern of nodulation at 32 C of Clark soybean with the five strains of R. japonicum gave a more flattened curve which was displaced downward relative to those at the lower temperatures. Nodulation generally was lower on the primary root, and some nodules were very far below the RTM. The pattern of nodulation obtained at this temperature looks much like that reported (Halverson and Stacey 1984, Heron and Pueppke 1984) for interactions with fewer nodules relative to other interactions tested in those studies. Heron and Pueppke (1984) reported a similar pattern on the soybean cultivar Vicoja inoculated with the fast growing R. japonicum strain 191. Halverson and Stacey (1984) reported that the delayed nodulating mutant strain HS111 produced a

similar scattered and downwardly displaced pattern on Essex soybean as compared to the pattern obtained with strain 110.

An explanation for the striking similarity in the nodulation pattern for all of these interactions is that the they are merely diagnostic for any interaction in which the initial number of successful infections is reduced and plant regulation of additional nodulation is not triggered (Pierce and Bauer 1983). That is, the similarities of the patterns may be coincidental. But the surprising similarity of those nodulation patterns to the ones in this study resulting from restrictive temperature, brings up the question as to what effect temperature might have on those interactions. The soybean cultivar Vicoja was developed at the Universidade Federal de Vicosa, Vicosa, Minas Gerais, Brazil, specifically for local Brazilian conditions, including high temperature. Thus the observed inefficiency of nodulation is perhaps due to assay temperatures below those to which Vicoja is adapted. Similarly, the possiblity that HS111 is a temperature sensitive mutant that has been tested only at restrictive temperatures cannot be ruled out.

The nodulation of Clark-*rj*$_1$ is so sparse that interpretation of the nodule profile is difficult. There is certainly no clear peak in the histogram near the point corresponding to the RTM. Whether such a peak would become evident if much greater numbers of plants were examined is uncertain, but seems unlikely; the pattern appears to be scattered and random. Although these data are insufficient

to either validate or invalidate the application of the model of Bhuvaneswari (1981) to Clark-$\underline{rj}_1$, these data show only limited correspondence with the expected pattern of nodulation predicted by the Bhuvaneswari model. The scattered and sparse nodulation and the reduced correlation of nodulation with the region near the RTM are likely to make the elucidation of early infection events in the nodulation restrictive $\underline{rj}_1\underline{rj}_1$-soybean that much more challenging.

CHAPTER FOUR
ADSORPTION OF STRAINS OF *RHIZOBIUM JAPONICUM* WITH DIFFERENTIAL NODULATING ABILITY TO ROOTS OF SOYBEAN ISOLINES THAT DIFFER AT THE Rj_1 LOCUS

Introduction

The ability of rhizobia to bind to roots of their legume hosts has long been assumed to have a principal role in the specificity of the infection process and is believed to be a major determinant of host range (Bhuvaneswari 1981, Dazzo 1981). These assumptions often are based on the correlation between binding of some component of a plant to a microbe *in vitro*, and the ability of that microbe to infect the plant (Dazzo and Hubbell 1975, Robertson et al. 1981). These and other indirect tests have been interpreted as evidence for a direct role of binding in determining host range of microsymbionts. The tests have included studies of cross-reacting antigens on plant and bacterial surfaces (Bishop et al. 1977, Dazzo and Hubbell 1975), the determination of number of nodules formed after various substances were added to plant roots with inoculum (Halverson and Stacey 1984), and tests for lectin binding to bacteria and the search for those lectins on or in plant roots (Bohlool and Schmidt 1974, Bhuvaneswari et al. 1977, Dazzo et al. 1978, Stacey et al. 1980, Gade et al. 1981, Law et al. 1982, Law and Strijdom 1984). Although these studies

have not always addressed the subject of bacterial binding to plant roots, they have been interpreted in terms of the role of adsorption. The underlying assumptions are i. that the examined process is a necessary antecedent to adsorption, or ii. that a tested factor has a direct intermediary role in adsortion. Unfortunately, these assuptions have not been tested.

Measurement of bacterial binding per se has been reported with mixed results in terms of its perceived role in host-range determination. Broughton et al. (1980) tested binding of ^{35}S-radioisotope-labelled strains of R. leguminosarum to roots of Pisum sativum. Their results are difficult to evaluate due to the very low specific activity of labelling attained and the high variability between experiments. They concluded that ability to adsorb to plant roots was not a determining factor in the differential ability of the bacteria tested to nodulate cultivars of pea. This conclusion was corroborated by microscopic examination of inoculated pea roots (Broughton et al. 1982). Chen and Phillips (1976) used ^{32}P-radioisotope-labelling of several rhizobia to test adsorption to severed root segments in vitro. Considerable radioactivity was taken up by the root segment tissues during incubation with the radioisotope-labelled bacteria. This fact and the artificiality of their assay conditions confound the conclusions. Their data tend to discredit the role of adsorption in host range

determination, because binding between their bacterial strains and plant roots was rather nonspecific.

Light microscopic examination of binding, either with transmitted light or fluorescence labelling techniques, has been used as an assay for adherence. From such studies qualitative rather than quantitative data are generally reported. Dazzo and Brill (1979) reported specific adherence of R. trifolii strain 0403 to root hairs of Trifolium repens. A strain of Azotobacter vinelandii that had been transformed with R. trifolii DNA and selected for antigenic cross-reactivity with T. repens also bound. They reported that an A. vinelandii revertant "did not adhere." Chen and Phillips (1976) reported that no differences were apparent between the binding of fluoresescent-labelled R. leguminosarum to roots of pea, which it normally nodulates, and the roots of several legumes which it does not nodulate.

Conversely, a direct role in adsorption has been inferred by Stacey et al. (1980) from their light microscopic and scanning electron microscopic examination of the binding of rhizobia to soybean (Glycine max) and wild soybean (G. soja). Unfortunately, they tested various haptens of lectins for their effect on binding but not on nodulation. The assays involved adsorption of rhizobia to the elongated root hairs, which are not believed to be normally infected in soybean (Bhuvaneswari 1981, Pueppke 1983, 1984a). Using similar binding assays in another study, Stacey et al. (1982) reported that, of a number of

mutant R. japonicum strains with genetic lesions affecting nodulation, only two failed to bind to soybean root hairs.

Two studies have measured bacterial binding to roots of soybean directly (Law et al. 1982, Pueppke 1984b). Law et al. (1982) determined the number of cells of mutant isolates which bound to 1 cm segments of excised soybean root. The root pieces were incubated for 1 hr in a dilute bacterial suspension, gently washed, ground, diluted, and plated. Between 1000 and 2300 bacteria bound per root segment. Unfortunately, nodulation cannot be compared to binding using inoculation conditions similar to those used in this assay since the binding assay uses severed roots. The loss of plant sap from the cut ends may also affect the number of bacteria bound. These problems were answered in the procedure devised by Pueppke (1984b), in which intact seedlings were suspended with their roots dangling in bacterial suspensions. After timed incubation the plants were removed and rinsed vigorously. A root segment was removed, ground, and plated for determination of colony forming units. Adsorption to seedling roots of soybean and cowpea by one fast-growing and four slow-growing rhizobia was independent of plant species and of the ability of the strains to nodulate these hosts. This procedure (Pueppke 1984b), although more cumbersome than the previous assay (Law et al. 1982), allows examination of root adsorption in a system similar to a commonly used inoculation protocol. The effect of the binding and rinsing conditions on

nodulation can be tested by treating seedlings and then transferring them to plastic growth pouches.

The objective of my research was to compare the binding of rhizobia to soybean roots, with the known abilities of the Rhizobium strains to nodulate specific genotypes of soybean (Devine 1984a). The study used the procedures developed Pueppke (1984b) to enable direct assay of bacterial binding to living plants. The assay conditions were similar to the customary inoculation procedure used for nodulation studies. Near-isogenic lines of soybean that have differential ability to form nodules with the R. japonicum strains were utilized. Two temperatures known to affect the number of nodules formed on these isolines (Devine and Breithaupt 1980b, Chapter Three) were tested.

Materials and Methods

The USDA strains 94 and 110 of Rhizobium japonicum were obtained from the USDA Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville, MD, courtesy of H. H. Keyser, D. F. Weber, and R. Griffin. They were maintained on yeast extract mannitol (YEM) agar (Vincent 1970) slants at 4 C. Cultures for adsorption studies were grown at 28 C with shaking in liquid defined gluconate-mannitol medium (Bhuvaneswari et al. 1977). Bacterial concentration was estimated turbidimetrically. Bacteria were pelleted by centrifugation at 7500 x g, washed once in sterile filtered nitrogen-free Jensen's plant growth solution (Vincent 1970), repelleted, and resuspended in Jensen's solution at

approximating 1 x 10^4 bacteria/ml. Aliquots were plated on YEM agar for determination of colony forming units to quantify viable bacteria in the inoculum.

Seeds of Glycine max (L.) Merr. cultivar Clark-L1 (Rj_1Rj_1) and its isoline, L63-1889 (rj_1rj_1), were obtained from R. L. Bernard, USDA Regional Soybean Laboratory, University of Illinois, Urbana, and D. A. Phillips, Department of Agronomy and Range Science, University of California, Davis. In this study the terminology of Devine and Breithaupt (1980b) is followed; the term Clark is used for the parent cultivar Clark-L1, and Clark-rj_1 designates the isoline L63-1889 carrying the nodulation-restrictive genes. The nodulation-restrictive genotype conditions resistance to nodulation by most strains of R. japonicum, including strain 110. Strain 94 is one of the "overcoming" strains which overcome rj_1-resistance and form a few nodules. Clark is nodulated abundantly by both strains.

Seeds were surface disinfested by soaking them in 50 percent ethanol for 2 min, rinsing, shaking in 0.5% aqueous sodium hypochlorite for 2 min, and rinsing in running deionized water for 20 min. Seeds were germinated on water agar for 4 d at 28 C in the dark. Seedlings lacking bacterial or fungal growth were placed, three per pouch, in autoclaved plastic growth pouches (Northrup King Seed Co, Minneapolis, MN) containing 15 ml of Jensen's solution. Pouches were covered with plastic sleeves to maintain sterility. Seedlings were placed under fluorescent lights

with a 12 hr light/dark cycle at 450 uE/m^2/sec irradiance for 1 d to allow root elongation.

The adsorption assays were completed as previously reported (Pueppke 1984b). All assays were done in a laminar flow hood under aseptic conditions. Bacterial inoculum (25 ml) was placed in each of sixteen 100 x 25 mm sterile test tubes. Two bent paper clips were hooked on each test tube rim, each to support a seedling. Seedlings were suspended with the roots immersed in the bacterial suspension. After 30, 60, 90, and 120 min, four sets of two seedlings were harvested. The roots of each seedling were washed vigorously with 25 ml of sterile filtered Jensen's solution delivered from a Brinkman Dispensette. The solution was delivered with the maximum possible stream that would still run down around the root when the seedling was held intersecting the stream. Each root was severed 2 cm from the root tip. The distal segments of the two roots from each tube were ground together in a ground-glass tissue grinder in 1 ml of sterile Jensen's solution. The resulting pulp was appropriately diluted and five 0.1 ml aliquots were plated on YEM agar plates. The plates were incubated in the dark at 28 C, and the colonies were counted after 7 to 10 d. In each experiment, for each plant type at each time, there were four replications represented by four tubes. Each experiment was repeated at least five times for each strain x plant combination.

The assays described above were completed at an ambient air temperature of approximately 27 C. Additional assays

were completed for the strain 94 x Clark-*rj*$_1$ combination at 22 C. All procedures were completed as described, except that the test tubes with inoculum were placed in a water bath and equilibrated to 22 C for 30 min before seedlings were added. Temperature was monitored carefully during the assay to maintain 22 $\pm$ 0.5 C.

Data were collected as the mean number of colonies formed on five replicate plates spread with suspensions resulting from grinding each set of root segments. The data were normalized to correct for experiment-to-experiment variation in actual inoculum density. This was accomplished by dividing treatment means by a ratio representing the turbidimetrically estimated inoculum (1×10^4) divided by the actual colony forming units in the inoculum. Normalized data are expressed as the number of bacteria adsorbed per plant.

Two kinds of control experiments were conducted. Known amounts of bacteria were ground with root tissue to determine the effect of grinding and of plant tissue constituents on numbers of bacteria producing colonies on YEM. The other control tested the effect of adsorption assay conditions on nodulation. Seedlings that had been incubated with bacteria were washed as described above and then directly placed into prepared growth pouches and maintained in a Conviron E-15 growth chamber at 22 C for 20 d with 900 uE/m^2/sec irradiance (400-700 nm) with a cycle 12 hr light and 12 hr dark.

Results

In the adsorption assay, both bacterial strains bound to Clark soybean roots in roughly similar numbers with a near linear increase with time. Approximately 100 bacteria were bound per plant at 2 hr (Figure 4.1). The capacity of Clark-*rj*$_1$ to adsorb overcoming strain 94 was very similar to that of Clark. Somewhat surprising was the binding of greater numbers of bacteria in the nonnodulating combination of Clark-*rj*$_1$ x strain 110. In this combination, the mean number of bacteria bound per plant was nearly 100 at 30 min, about twice the number bound in the Clark x 110 combination.

A reduction in assay temperature from 27 to 22 C markedly decreased the binding of strain 94 to the isoline Clark-*rj*$_1$ (Figure 4.2). After 1 hr the number bound at 27 C was 59 $\pm$ 4 ($\pm$ SE), but at 22 C was 15 $\pm$ 1, about a 75% reduction. After 2 hr, the number bound at 27 C was 105 $\pm$ 8 but for 22 C was 21 $\pm$ 2, an 80% reduction. The effect of these temperatures on adsorption was opposite to their effect on nodulation. At 22 C this plant-strain combination had a greater number of nodules, a greater percentage of the plants with nodules, and a greater number of nodules on the primary root than at 27 C (Tables 4.1 and 4.2 [data from Chapter Three]).

Controls in which inoculum with known numbers of bacteria was ground with either type of root tissue produced colony counts which were not significantly different from

each other or from those plated directly from the inoculum. Controls to test the suitability of the conditions of the adsorption assay for nodulation were examined 20 d after placing seedlings in growth pouches. The seedling had been incubated in inoculum for 120 min and washed in parallel treatments to seedlings in adsorption assays. The mean number of nodules per plant for each combination is presented in Table 4.3. Only the combination of strain 110 x Clark-$\underline{rj}_1$ and the seedlings incubated in sterile Jensen's solution without bacteria failed to form nodules. The following number of plants were nodulated in the other combinations: Clark-$\underline{rj}_1$ x strain 94, 1 of 8; Clark x strain 110, 8 of 8; and Clark x strain 94, 8 of 8. Thus, it is clear that adsorption was assayed under conditions which were conducive to infection leading to nodulation.

Discussion

One objective of this study was to determine whether the nodulation restrictive genotype, Clark-$\underline{rj}_1$, reduces the ability of the plants to adsorb nonovercoming strains of Rhizobium, thus influencing bacterial host range. This is not the case, because after 2 hr Clark-$\underline{rj}_1$ adsorbed more of either R. japonicum strain tested that did Clark. In fact, the ranking of plant type x strain combinations based on numbers of nodules is precisely opposite to their ranking by numbers of bacteria adsorbed at 2 hr. Although no biological significance is apparent, this inverse

Figure 4.1. Adsorption of cells of Rhizobium japonicum to soybean roots. The experiments were completed at 27 C. Each point represents the mean from five experiments with four pairs of plants tested at each time for each plant-strain combination at each replication of the experiment. The roots of soybean seedlings were incubated in bacterial suspensions (1 x 10^4 cells/ml) for the times indicated and rinsed vigorously. The terminal 2 cm of the primary roots were excised, ground and plated for determination of colony forming units. □ = Clark x strain 110, ■ = Clark x strain 94, ○ = Clark-rj_1 x strain 110, and ● = Clark-rj_1 x strain 94.Bars represent the standard error of the mean.

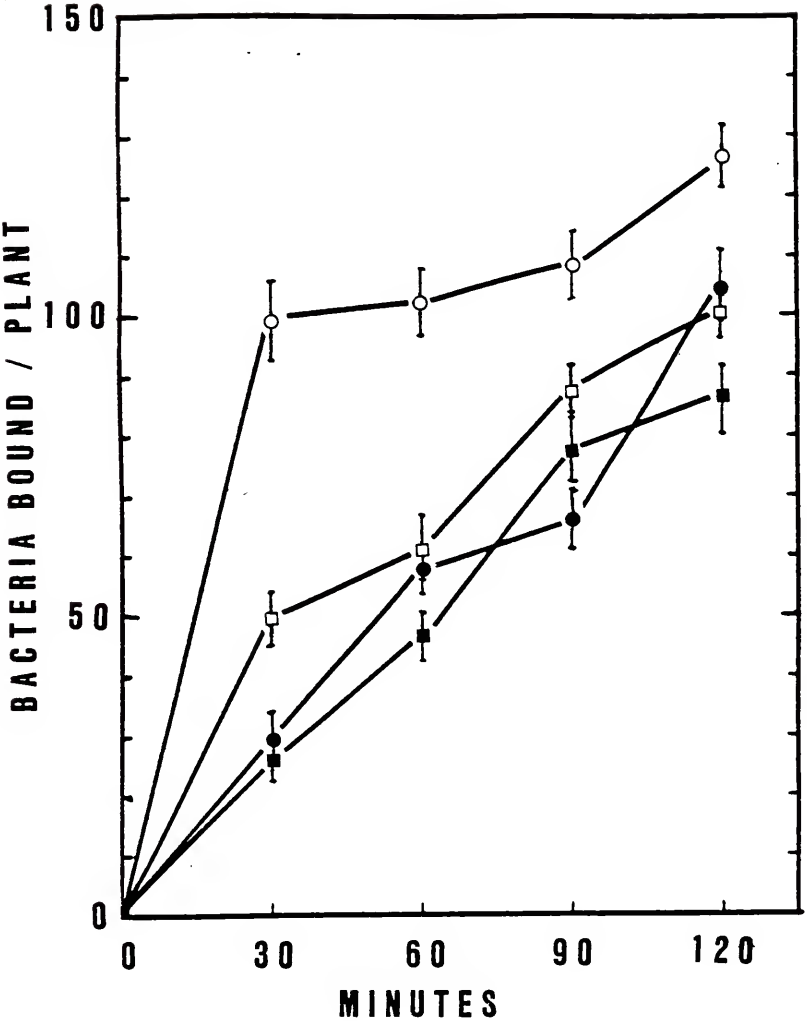

150
100
50
0
BACTERIA BOUND / PLANT
0
30
60
90
120
MINUTES

Figure 4.2. Adsorption of cells of Rhizobium japonicum strain 94 to roots of Clark-rj_1 soybean at two temperatures. Each point represents the mean of four pairs of plants tested at each time for each temperature from each of five replications of the experiment. The roots of seedlings were incubated in bacterial suspensions (1 x 10^4 cells/ml) equilibrated at 22 C or 27 C for the times indicated and rinsed vigorously. The terminal 2 cm of the primary roots were excised, ground and plated for determination of colony forming units. ∇ = 22 C, and ● = 27 C (The 27 C curve is duplicated from Figure 3.2.). Bars represent the standard error of the mean.

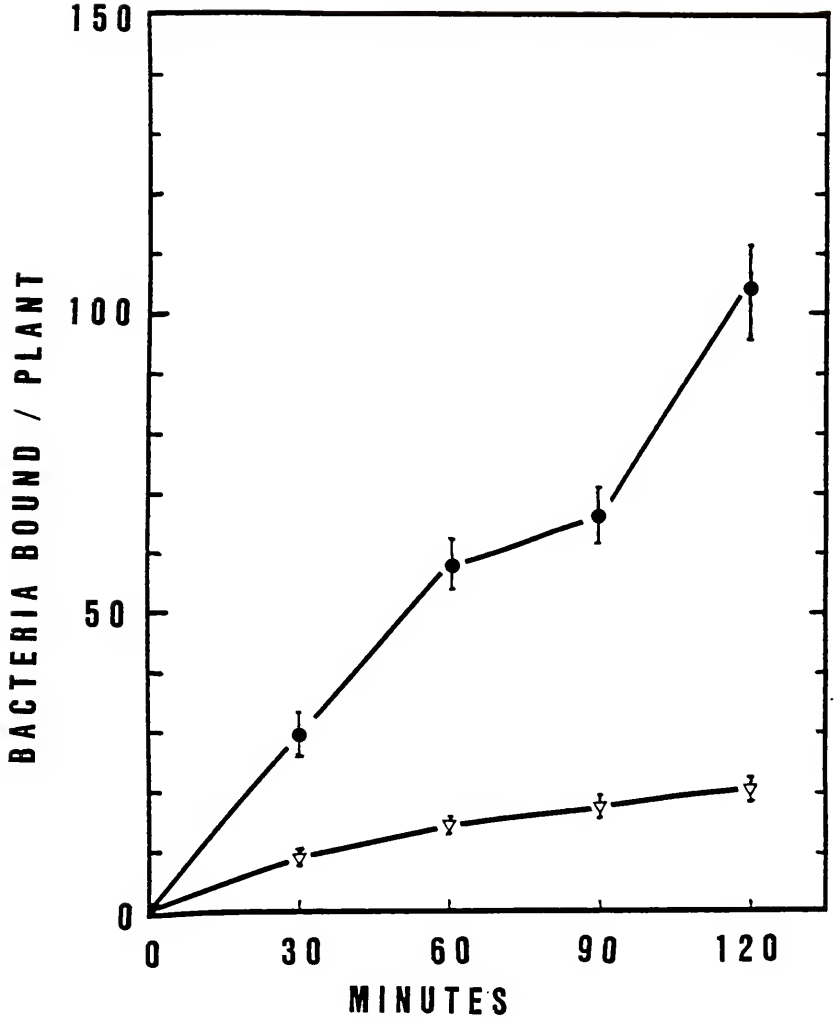

150
100
50
0
BACTERIA BOUND / PLANT
0
30
60
90
120
MINUTES

Table 4.1. Percentage of plants nodulated at 27 C and 22 C

Combination	Primary		Secondary		Any Root	
	27 C	22 C	27 C	22 C	27 C	22 C
Clark x 94	89[a]	100	89	100	100	100
Clark x 110	89	100	50	89	100	100
Clark-*rj*$_1$ x 94	0	13	10	27	10	40
Clark-*rj*$_1$ x 110	0	0	0	0	0	0

[a] Data in table from Chapter Three. Percentage of plants nodulated at each indicated location. Data are for all plants from 3 replications at each temperature with 6 to 10 plants per replication.

Table 4.2. Mean number of nodules per plant at 27 C and 22 C

Combination	Primary		Secondary		Any Root	
	27 C	22 C	27 C	22 C	27 C	22 C
Clark x 94	8.8[a]	7.9	5.8	10.2	14.6	18.1
Clark x 110	8.3	8.9	3.6	6.8	11.9	15.7
Clark-*rj*$_1$ x 94	0	0.3	0.1	0.5	0.1	0.9
Clark-*rj*$_1$ x 110	0	0	0	0	0	0

[a] Data in table from Chapter Three. Mean number of nodules per plant on primary roots, secondary roots, or on any root of the plant as indicated. Each value represents the average for 3 replications at each temperature with 6 to 10 plants per replication.

Table 4.3. Nodulation of plants inoculated with *Rhizobium japonicum* under the conditions of the adsorption assay

Soybean isoline	Bacterial strain 94	110
Clark	8.3 ± 5.7[a]	6.5 ± 2.1
Clark-*rj*$_1$	0.1 ± 0.4	0

[a] Seedlings were inoculated by dipping the roots in a bacterial suspension containing approximately 10^4 cells/ml for 2 hr. The roots were rinsed vigorously. Plants were grown for 20 d in plastic growth pouches (see Materials and Methods for detailed description of procedures).

relationship underscores the rejection of the hypothesis that host range is primarily dependent on ability of rhizobia to adsorb to host roots, at least for the differential nodulation of Clark and its isoline, Clark-$\underline{rj_1}$.

Clark (1957) reported that similar numbers of rhizobia were recovered from the roots of plants carrying the $\underline{rj_1 rj_1}$ genotype and plants that carried a nonrestrictive genotype whether they were grown in a greenhouse or in the field. Elkan (1962) demonstrated that Clark-$\underline{rj_1}$ actually maintained substantially higher populations of rhizobia in rhizosphere soil in the field than did Clark for 45 out of the first 60 d of plant growth. These observations, in conjunction with the results of the present study, suggest that the limiting step in nodulation of these plants occurs post-adsorption.

Pueppke (1984b) showed that adsorption of R. japonicum strain 138 to roots of the soybean cultivar Hardee is temperature sensitive; when that combination was subjected to assay temperatures of 4 C, 27 C, and 37 C, the optimum binding temperature was 27 C. The number of bacteria bound per plant after co-incubation for 1 hr was reduced approximately 90% at 4 C and approximately 65% at 37 C, compared to the number bound at 27 C. In the present study, the binding of strain 94 to roots of Clark-$\underline{rj_1}$ similarly was temperature-sensitive. The reduction in the number of bacteria bound per plant in 1 hr with a drop in assay temperature from 27 C to 22 C was about 75%. The large reduction in adsorption with only a 5 degree temperature

difference suggests that this combination is either more highly temperature sensitive than the combination used in the previous study (1984b), or that perhaps the curve of temperature sensitivity for both combinations is very steep on either side of an optimum temperature. The shape of the response curve has not yet been determined, and would require testing adsorption at considerably more temperatures than have been used as treatments in either report.

For the combination of Clark-$\underline{rj}_1$ with strain 94, the response of adsorption to temperature is opposite to that of nodulation to temperature for the same (Tables 4.1 and 4.2 [data from Chapter Three], Devine and Breithaupt 1980b). Although 40% of the plants were nodulated at 22 C, only 10% were nodulated at 27 C. The number of bacteria bound per plant after 2 hr at 22 C was 21 $\pm$ 2. At 27 C 105 $\pm$ 8 bacteria were bound, a five-fold increase. Clearly the temperature effect on nodulation of these plants is independent of its effect on binding.

The following conclusions are drawn from the data presented in this report: i. Compared to 27 C, 22 C favors nodulation in the Clark-$\underline{rj}_1$ x strain 94 combination, whereas, the effect of temperature on adsorption is precisely the opposite. ii. There is not a qualitative difference between the adsorption of strains 94 and 110 to Clark and Clark-$\underline{rj}_1$ soybean roots. iii. The rates of adsorption in these combinations are similar to the rates reported for other strain x soybean cultivar combinations

(Pueppke 1984b). iv. Under these experimental conditions, there is no correlation between the number of rhizobia bound to roots and the extent of nodulation of Clark or Clark-rj_1 soybean.

CHAPTER FIVE
INFECTION OF SOYBEAN ISOLINES DIFFERING AT THE Rj_1 LOCUS BY *RHIZOBIUM JAPONICUM* STRAINS WITH DIFFERENTIAL NODULATING ABILITY

Introduction

The nodulation-restrictive genotype of soybean, rj_1rj_1, originated as a field mutant in a soybean breeding program (Williams and Lynch 1954). The phenotype was first characterized as nonnodulating with *Rhizobium japonicum* (Williams and Lynch 1954), but Clark (1957) reported that a few strains, called overcoming strains, form a few nodules on plants growing in sand or vermiculite but not soil. Several investigators have sought to identify the factor that differentiates rj_1rj_1-plants from those that nodulate normally. The amino acid content of both plant types has been compared and found to be similar (Clark 1957, Hubbell and Elkan 1967b). The restrictive step is not the inability of the rj_1rj_1-plants to support rhizobia in the rhizosphere; such plants support populations equal to (Clark 1957) or greater than (Elkan 1962) those supported by a near-isogenic but normally nodulating soybean line. Devine and Weber (1977) determined that successfully nodulated rj_1rj_1 plants were capable of fixing nitrogen. From these studies, Devine (1984a) inferred that the incompatibility conditioned by the

$\underline{rj_1rj_1}$ genotype is not a general antagonism to Rhizobium metabolism and function, but that the answer to incompatibility is likely to be found in early stages of the infection process.

A number of investigators have attempted to elucidate the bacterial property that enables a few strains to overcome the plant's resistance and produce some nodules. Hubbell and Elkan (1967a) reported that several physiological characteristics of R. japonicum strains were correlated with the ability to nodulate $\underline{rj_1rj_1}$-soybean, but none of the factors was implicated in infection. Devine and Weber (1977) noted that the ability of bacteria to nodulate the $\underline{rj_1rj_1}$ genotype was highly correlated with the production of a bacterial metabolite that induces chlorosis in several soybean lines. The bacterial product was latter shown to be rhizobitoxine (2-amino-3-hydroxypropoxy-vinylglycine) (Owens et al. 1972). They postulated that this chlorosis-causing agent had an enabling role in infection of $\underline{rj_1rj_1}$-soybean. Devine and Breithaupt (1980b) showed that processes leading to nodulation and to the expression of chlorosis had different temperature optima. When added to a Rhizobium inoculum, an analog of rhizobitoxine did not enhance nodulation of the restrictive soybean (Devine and Breithaupt 1980a). No diffusible compound was detected in tests for a factor to endow $\underline{rj_1}$-incompatible strains with the ability to nodulate the $\underline{rj_1rj_1}$-soybean (Devine et al. 1981). Devine concluded that the correlation of rhizobitoxine-induced chlorosis and

ability to form nodules on the restrictive soybean is likely incidental and "not the result of an intrinsic physiological relationship" (Devine 1984a [p 150]).

Infection of the small-seeded temperate legumes has been studied in the most detail due to the development of the Fahraeus slide technique enabling study of the root surface of living plants with the light microscope (Nutman 1981). These plants, which are are nodulated by fast-growing rhizobia, are believed to be infected exclusively through infection threads in root hairs (Fahraeus 1957, Nutman 1959, Ljunggren 1969, Callaham and Torrey 1981). Some tropical legumes, such as peanut (Arachis hypogaea L.) and several species of Stylosanthes, are infected through natural wounds caused by emergence of lateral roots, with no infection thread formation in root hairs (Allen and Allen 1940, Ranga Rao 1977, Chandler 1978, Chandler et al. 1982). In the tropical genus Lotus, one species (L. corniculatus L.) was reported to be infected only by means of infection threads in root hairs, but in another (L. hispidus Desf.) most nodules originated by bacterial penetration directly through the epidermis, and infected root hairs were rare (Ranga Rao 1977).

In 1938, Bieberdorf described the infection process in soybean in considerable detail. He was the first to note that infections usually progress via infection threads in soybean, but he states that rhizobia also infect directly through root epidermal cells. Apparently, corroboration of

the initial report of root hair infection of soybean was not made until fifty years later (Ranga Rao and Keister 1978) and direct infection processes have not been corroborated. Several studies of nodule structure have included some details of early infection (Cabezas de Herrera and Fernandez 1982, Goodchild and Bergersen 1966, Newcomb et al. 1979). Pueppke (1983) recently examined soybean in nodulating and nonnodulating combinations with eighteen strains of rhizobia for signs of early infection. He showed that infection threads were formed exclusively in nodulating combinations and that infection threads were restricted to locations distal to the region of the root on which root hairs were fully elongated at the time of inoculation. A thorough description of root hair infection at the ultrastructural level was recently reported by Turgeon and Bauer (Turgeon and Bauer 1982, In press).

Near-isogenic lines of soybean were examined in this study to determine whether the difference between the phenotypes of normally nodulating soybean and nodulation-restrictive ($\underline{rj_1}\,\underline{rj_1}$) soybean were expressed at the level of infection. Both Nutman (1981) and Devine (1984a) have suggested that the $\underline{rj_1}\,\underline{rj_1}$-soybean might be infected exclusively by means other than infection threads in root hairs. The mode of infection of $\underline{rj_1}\,\underline{rj_1}$-soybean has not previously been reported.

Materials and Methods

The bacteria utilized were *R. japonicum* strains 94 and 110 obtained from the U. S. Department of Agriculture Nitrogen Fixation and Soybean Genetics Laboratory, Beltsville, MD, courtesy of H. H. Keyser, D. F. Weber, and R. Griffin. Strain 94 is an overcoming strain that forms a few nodules on the nodulation-restrictive isoline, but forms abundant nodules on Clark (Chapter Three). Strain 110 nodulates Clark abundantly but does not nodulate the restrictive isoline (Chapter Three). Rhizobia were maintained on yeast extract-mannitol (YEM) agar (Vincent 1970) slants at 4 C, and stock cultures were transferred about every three months. Bacteria for inoculation were grown to mid-log phase at 28 C with shaking in 50 ml liquid defined gluconate-mannitol medium (Bhuvaneswari et al. 1977). Cell number was estimated turbidimetrically. Cultures then were centrifuged at 7500 x g for 10 min and bacteria were resuspended at a concentration of approximately 5 x 10^8 cells/ml (except where noted) in sterile Jensen's solution (Vincent 1970).

The plants were two near-isogenic lines of soybean *Glycine max* (L.) Merr., the cultivar 'Clark-L1' (Rj_1Rj_1) and its isoline L63-1889, which carries the nodulation-restriction genotype, rj_1rj_1. The isolines will be referred to as Clark and Clark-rj_1, respectively, following the terminology of Devine and Breithaupt (1980b). Seeds were obtained from R. L. Bernard, USDA Regional Soybean Laboratory, University of Illinois, Urbana, and D. A.

Phillips, Department of Agronomy and Range Science, University of California, Davis. Seeds were soaked in 50% aqueous ethanol for 2 min with agitation, rinsed with water, soaked in 0.5% aqueous sodium hypochlorite for 2 min with agitation, and rinsed for 20 min under running deionized water. Surface disinfested seeds were germinated at 22 C in the dark for 5 d on water agar plates.

Inoculation and seedling transfer were completed in a laminar flow hood using procedures designed to maintain sterility. Germinated seedlings were carefully selected for lack of any sign of contaminating microorganisms and for uniform size (ca. 4 cm, excluding cotyledons). Seedlings were inoculated by immersing the roots in the bacterial suspension for 10 min. Seedlings were placed, 2 per pouch, in plastic growth pouches (Northrup King Seed, Co., Minneapolis, MN). Growth pouches had been prepared by adding 15 ml nitrogen-free Jensen's plant growth solution to each and autoclaving. Control plants were sham-inoculated with sterile Jensen's solution only. A mark, called the root tip mark (RTM), was made on the growth-pouch surface at the position of the primary root tip. Except where noted, the tops of growth pouches were covered with plastic sleeves for several days to prevent contamination. Plants were grown at 22 C in a Conviron E-15 growth chamber at an irradiance of 900 uE/m^2/sec (400-700 nm) with 12 hr light, 12 hr dark.

Root surfaces were examined by light microscopy for curled root hairs of the type referred to previously as "question mark shaped" (Pueppke 1983), and for the presence of infection threads. Plants used in light microscopy experiments were grown essentially as stated above, except that seeds occasionally were germinated and plants grown at other temperatures and light intensities. Growth pouches were not covered during plant growth. Plants were sampled for light microscopy as previously described (Pueppke 1983) by stripping away very thin strips of tissue from root surfaces for several centimeters on either side of a point corresponding to the RTM. Two to four strips were made for each plant. These collectively included almost all of the root surface in the region of the RTM. Root surfaces of 18 Clark plants inoculated with strains 94 or 110, and 27 plants of Clark-*rj1* inoculated with strain 94 were examined. These strips were mounted in phosphate-buffered saline (Bhuvaneswari et al. 1977) with or without prior staining with toluidine blue O and were examined using bright-field or interference-contrast optics (Hoffman modulation). The entire strips were scanned for short, curled root hairs and infection threads. Light microscopy also was used to examine the surface of nodules for residual curled root hairs and infection threads. Plants were examined for very young developing nodules. The nodules were removed from the plants and a thin layer of the nodule surface distal to the plant was sliced away with a sharp scalpel. These thin

disks of tissue were mounted and examined as described for root surface strips.

Samples for scanning electron microscopy (SEM) were obtained from 10 day-old plants by severing the primary root with a scalpel at the RTM and at 10 mm above the RTM. Secondary roots attached to these sections were severed at a line parallel to the primary root, at 5 mm perpendicular from the primary root. The root sections immediately were placed into a fixing solution consisting of 4% glutaraldehyde (Eastman Kodak Co.) in 50 mM sodium cacodylate buffer (pH 7.4). After fixation overnight at 4 C, samples were rinsed with distilled water and post-fixed overnight at 4 C in 2% aqueous osmium tetroxide. Sections were washed in 5 changes of distilled water and dehydrated progressively in a graded water-ethanol series with never more than a 10% increase in ethanol concentration per step or less than 15 min per step. A faster dehydration or steeper series caused distortion. The samples were transferred in anhydrous ethanol to a critical point dryer (Balzers Model H) and dried from liquid carbon dioxide. Dried samples were attached to Cambridge mounts (Ernest F. Fullam, Inc.) with double-sided tape, sputter-coated with ionized gold, and examined with a Hitachi S-450 scanning electron microscope at 20 kV.

Two or more groups of 5 plants were sampled and fixed separately for each combination of Clark x strain 110, Clark x strain 94, and Clark-$\underline{rj}_1$ x strain 94. Additional plants were examined for some combinations. Two uninoculated Clark

control plants and two of the Clark-$\underline{rj}_1$ x strain 94 combination that had been inoculated at 1 x 10^{10} cells/ml were examined. Root sections were examined using SEM at low magnification (50x) and photomicrographs were made of the entire root segment to enable the mapping of higher magnification photomicrographs for interpretation in context. Nodule surfaces and root surfaces were examined. All samples were scanned at 150x and 250x for curled root hairs of the type previously identified by light microscopy to harbor infection threads.

Results

Clark soybean inoculated with either of the fully compatible strains 110 or 94 at 5 x 10^8 cells/ml had short, tightly curled root hairs (Figure 5.1), many containing infection threads. The tightly curled root hairs with infection threads sometimes occurred in clusters. A number of root surface strips were examined from 27 plants for the combination Clark-$\underline{rj}_1$ x strain 94, which was previously demonstrated to produce a few nodules (Chapter Three). No short, tightly curled root hairs similar to those seen in the fully compatible combinations were found and no infection threads were ever observed.

Light microscopy was used to examine nodule surfaces for residual root hairs with infection threads. Thirteen of twenty sections of nodule surface tissue examined for the

Figure 5.1. Curled root hair on Clark soybean 10 days after inoculation with Rhizobium japonicum strain 110. Phase contrast optics.

Clark x strain 94 combination had curled root hairs, and in 9 the infection thread(s) was still visible. None of twelve nodules examined similarly from the Clark-$\underline{rj}_1$ x 94 combination had curled root hairs or visible infection threads.

Primary root segments extending from the RTM to 10 mm above the RTM were examined using SEM. Uninoculated plants developed long root hairs that were free of detectable bacteria (Figure 5.2). No short, curled root hairs were apparent except where it was obvious that the root had come in contact with a solid surface such as the plastic of the growth pouch. In these areas the individual deformed root hairs were easily discriminated from root hairs characteristic of inoculated roots of compatible combinations. Root segments from Clark soybean inoculated with 5 x 10^8 cells/ml of fully compatible strains of R. japonicum were examined using SEM. The root segments from Clark soybean inoculated with strain 110 had clusters of short curled root hairs similar in appearence to the root hairs shown by light microscopy to contain infection threads (Figure 5.3). Clark inoculated with strain 94 produced short curled root hairs very similar to those seen in the the combination Clark x strain 110 (Figures 5.4 and 5.5). There were differences in the general appearence of root segments from these two combinations. The root surfaces of Clark were smooth (almost waxy appearing in SEM micrographs)

Figure 5.2. Clark soybean root hairs on a control plant sampled and fixed 10 days after sham inoculation with plant growth solution. The area sampled was the 10 cm of the primary root immediately above the point representing the root tip at the initiation of the experiment.

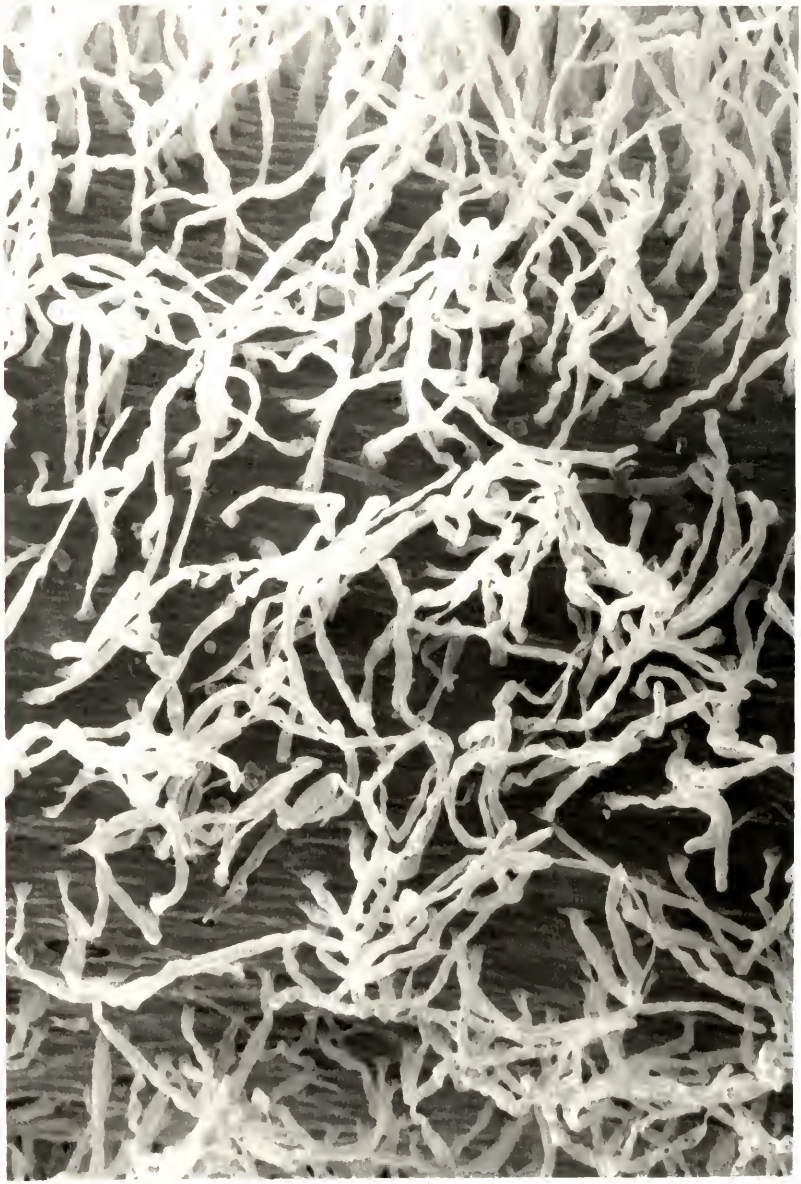


Figure 5.3. Clark soybean root hairs on plants sampled 10 days after inoculation with Rhizobium japonicum strain 110. Clusters of tightly curled root hairs characteristic of this plant-strain combination are marked by arrows.

Figure 5.4. Curled root hair on Clark soybean sampled 10 days after inoculation with Rhizobium japonicum strain 110. Note the bacterial cells adsorbed to the root hair and epidermal surface.

Figure 5.5. Curled root hair on Clark soybean sampled 10 days after inoculation with Rhizobium japonicum strain 94.

in the combination with strain 110, whereas when inoculated with strain 94, Clark roots often had a roughened appearance in localized patches that frequently were associated with presence of bacteria (Figure 5.6). Remnants of tightly curled root hairs were seen with SEM on surfaces of nodules produced from both interactions (Figure 5.7).

Clark-$\underline{rj}_1$ roots which had been inoculated with strain 94 (5 x 10^8 cells/ml or 10^{10} cells/ml) were examined using SEM. No short, tightly curled root hairs of the type observed in the fully compatible interactions of Clark with strains 94 and 110 were seen for either of these treatments. Roughened areas were observed on root surfaces inoculated with the lower cell concentration similar to those noted for the combination Clark x strain 94. For Clark-$\underline{rj}_1$ soybean which had been inoculated with strain 94 at 10^{10} cells/ml, there was evidence of degradation of a surface component, presumably mucigel (Foster et al. 1983), associated with the presence of bacteria on the root surface (Figure 5.8). The cell walls of some epidermal cells appeared to be perforated (Figures 5.9, 5.10, and 5.11). These perforations usually were heavily populated with bacteria. The perforations were most often seen at the base of short, uncurled root hairs; yet some epidermal cells which had no evidence of root hair development were apparently perforated and colonized by bacteria. The breached epidermal cells were associated with those areas where roughening of the mucigel-like material was most evident.

Figure 5.6. A tightly curled root hair is seen at center on Clark soybean root surface 10 days after inoculation with Rhizobium japonicum strain 94. The arrows indicate roughened areas on root surface associated with inoculation with R. japonicum strains possesing the ability to overcome the rj_1rj_1-resistance to nodulation.

Figure 5.7. Residual root hairs on the Clark soybean nodule surface 10 days after inoculation. One of the root hairs is tightly curled (arrow).

Figure 5.8. Note the extensive colonization of the root surface by bacteria and the degradation of a surface component, presumably mucigel (arrows). Clark-rj$_1$ soybean root surface sampled 10 days after inoculation with Rhizobium japonicum strain 94 (10^{10} cells/ml).

Figure 5.9. Extensive colonization of the root surface of Clark-*rj*$_1$ by bacteria 10 days after inoculation with *Rhizobium japonicum* strain 94 (10^{10} cells/ml). Apparent degradation and penetration of the epidermal cells is apparent (arrows). The boxed areas are shown at higher magnification in the following two figures.

Figure 5.10. Clark-rj_1 soybean root surface sampled 10 days after inoculation with *Rhizobium japonicum* strain 94 (10^{10} cells/ml). Arrows indicate locations of apparent penetration of the epidermal cells. This view is a higher magnification of an area shown in Figure 5.9.

Figure 5.11. Clark-rj_1 soybean root surface sampled 10 days after inoculation with Rhizobium japonicum strain 94 (10^{10} cells/ml). Arrows indicate locations of apparent penetration of the epidermal cells. This view is a higher magnification of an area shown in Figure 5.9.

Discussion

When Clark soybean was inoculated with either of the fully compatible strains 110 or 94 (5 x 10^8 cells/ml), clusters of tightly curled, short root hairs were formed by the tenth day of growth. These curled root hairs were detected both by light microscopy of thin strips of root surface tissues and by SEM of root segments. They were similar to those described by Pueppke (1983) as resembling "question marks." As reported in the previous study (Pueppke 1983), many of the question mark-shaped root hairs contained infection threads readily resolved at a magnification of 400x with either bright field or interference contrast optics (Hoffman modulation). Scanning electron micrographs were produced of the tightly curled root hairs for both of these compatible combinations. The curled root hairs appeared similar to those represented by the scanning electron micrographs of the compatible strain 110 x Williams combination (Turgeon and Bauer In press). In my study, the tightly curled hairs often were free-standing (Figures 5.4 and 5.5), although curled over almost to the epidermal surface or even touching it. Root hairs that were tightly appressed to the epidermal cells (Turgeon and Bauer In press) were less common.

Ranga Rao and Keister (1978) published light micrographs of infected root hairs of the soybean cultivar Beeson infected by several Rhizobium strains. Some of their micrographs showed the tightly curled infected root hairs of

the type exclusively seen in this study and in other reports (Pueppke 1983, Turgeon and Bauer In Press), but most showed elongated root hairs with curling only at the ends with the infection threads clearly visible. Turgeon and Bauer (1982) saw only the short, tightly curled hairs when they examined that same cultivar with a different strain. Therefore, it seems more likely that the growth conditions rather than specific cultivar x strain interactions induce the different response. Ranga Rao and Keister (1978) used a hydroponic culture, as have the other studies (Turgeon and Bauer 1982, In Press, Pueppke 1983), but they used sand as a support medium rather than the paper support used in the growth pouch system. Bieberdorf (1938) also reported that infections occurred in elongated root hairs. He claimed, additionally, that those elongated root hairs with infection threads were uncurved. But, if the illustration he cites as showing such an infection (Bieberdorf 1938 [Figure 1.a]) is compared with his other illustrations of infection, it can be conjectured that the root hair he describes as "without curvature," is actually curved out of the focal plane (Compare also Pueppke 1983 [Figure 5]). Although he includes photomicrographs in his report, illustrations showing infection all were produced by means of a camera lucida. Perhaps the lack of sufficient depth-of-field and resolution of his microscope, and the extra simplification created by the camera lucida, caused him to overlook curling which was actually present. No other report has claimed infection of soybean in fully elongated root hairs without

curvature. The other illustrations of infection of soybean that Beiberdorf provides are in general agreement with this study.

No tightly curled root hairs of the type shown to have infection threads in nodulating combinations were observed on strips of root epidermis from Clark-$\underline{rj}_1$ plants inoculated with strain 94. The procedures in which root surfaces were examined for infection structures might not be adequate to detect a rare event. For instance, since the average is less than one nodule formed per Clark-$\underline{rj}_1$ plant, if Clark-$\underline{rj}_1$ were infected through root hairs by infection threads but only one infection occurred per nodule, that infected root hair would be unlikely to be observed. Since investigators of early infection processes in soybean have noted curled root hairs on surfaces of expanding nodules (Bieberdorf 1938, Ranga Rao and Keister 1978), and since nodules are the only marker for a successful infection, it is logical to use them as a means to localize the search for root hair infection. When free-hand sections of nodules formed by strain 94 were examined microscopically, curled root hairs with infection threads were found on nodules from Clark, but curled root hairs were not observed on nodules from Clark-$\underline{rj}_1$. I provisionally conclude that Clark-$\underline{rj}_1$ is not infected through root hairs as has been reported for other soybean genotypes. It appears that $\underline{rj}_1\underline{rj}_1$-resistance to infection by most strains of rhizobia is expressed as resistance to infection via root hairs.

La Favre and Eaglesham (1981) suggested in a preliminary report that a very high inoculum concentration could increase the nodule number on sand-grown $\underline{rj_1}\underline{rj_1}$-soybean. The effect in this study of increasing the concentration of strain 94 to approximately 1 x 10^{10} cells/ml for inoculation of Clark-$\underline{rj_1}$ was observed using SEM. The extensive roughening of the root surface and the apparent perforation of epidermal cells in Clark-$\underline{rj_1}$ plants suggests enzymatic degradation of plant surface and cell wall components resulting from inoculation. It appears, therefore, that the ability of overcoming strains to infect $\underline{rj_1}\underline{rj_1}$-soybean could be due to their ability to penetrate epidermal cells directly. Such an infection mechanism has been described for several other legumes (Chandler et al. 1982, Ranga Rao 1977). Several rhizobia are known to produce the potential wall degrading enzymes cellulase and pectolytic enzymes (Hubbell et al. 1978, Morales et al. 1984). The earliest desciption of soybean infection included direct infection through epidermal cells as one of the alternative infection mechanisms, but that report (Bieberdorf 1938) has not been corroborated and often has been discounted. An alternative to root hair infection, such as direct penetration, would potentially explain the anomalous nodulation of Clark soybean in the zone of mature root hairs by the overcoming strains of R. japonicum (Chapter Three), assuming that the alternative pathway of direct infection is occurring at a low frequency and without the developmental constraints observed for root hair

infection. Such anomalous nodulation was not observed for strain 110, which correlates with its inability to overcome the $\underline{rj_1}\,\underline{rj_1}$-resistance and presumed inability to infect by direct penetration.

I should quickly add the caveat that these observations, though they suggest direct penetration as an infection mechanism for the overcoming strains, are merely correlative and not direct evidence for such a mechanism. Compelling evidence for such a mechanism would be provided by genetic alteration of the bacterium affecting a single gene which caused either a gain or loss of both the ability to infect $\underline{rj_1}\,\underline{rj_1}$-soybean and the ability to penetrate soybean root surfaces directly. Persuasive evidence for direct penetration leading to infection could also be obtained though transmission electron microscopy, as it was for infection mechanisms of peanut and Stylosanthes (Chandler 1978, Chandler et al. 1982). No simple procedure has yet been devised to localize infections leading to nodules in Clark-$\underline{rj_1}$ before the actual appearance of those nodules. That difficulty is underscored by the amount of effort that has been invested with incipient infections being located (Tanner and Anderson 1963).

The suggested additional infection pathway for overcoming strains--through direct penetration--is one more distinguishing characteristic, in addition to those noted by Devine (1984a), between these rhizobia and the other strains that infect soybean. He suggests, based on several lines of

evidence, that the overcoming strains constitute a separate genetic population that should be separated phylogenetically from the other strains now included in R. japonicum. My conclusion that the $\underline{rj_1}\underline{rj_1}$-genotype of soybean is characterized as lacking infection through root hairs but is nodulated, albeit sparsely, by certain strains of R. japonicum, leads to a unique description of the symbiotic phenotype. Following the terminology of Vincent (1980), the interaction of Clark-$\underline{rj_1}$ with the overcoming strain 94 would be characterized as Inf^- (no infection threads formed) Nod^+ (nodules formed). All other soybean x Rhizobium combinations that have been characterized have been either Inf^- Nod^- or Inf^+ Nod^+. This is the first report of a combination characterized as Inf^- Nod^+.

CHAPTER SIX
SUMMARY

The interaction phenotype was examined for the soybean isolines, Clark and Clark-$\underline{rj}_1$, with strains of Rhizobium japonicum possesing differential ability to nodulate. Various factors were examined for their role in the infection and nodulation process. The effect of temperature on nodulation was tested to find optimum conditions for study of infection processes. Temperature had statistically significant effects on nodulation of both plant types irrespective of bacterial strain. The largest number of nodules per plant and the highest percentage of nodulated plants was at 22 C. When inoculated Clark plants were grown at 32 C, nodulation on the primary root was displaced downward and was more random. The nodule profile lacked a definite peak representing nodule production near the location of the root tip of the primary root at inoculation. This peak was present for plants grown at 27 C and 22 C.

The role of adsorption of rhizobia to roots of the soybean isolines was tested for strains with and without the ability to overcome the Clark-$\underline{rj}_1$ resistance to nodulation. When isoline x strain combinations were ranked by the number of bacteria bound to roots, the order was opposite to their rank by nodules formed per plant. Thus the hypothesis that

the differential ability of the two strains to nodulate Clark-$\underline{rj}_1$ soybean was mediated by selective adsorption to plant roots was rejected. Reduction of temperature from 27 C to 22 C reduced the number of bacteria bound to roots of Clark-$\underline{rj}_1$ by 20%. This is precisely opposite the effect of those temperatures on nodule number. Clearly, the temperature effect on nodulation also occurs at some step subsequent to adsorption of bacteria to roots.

Roots of the soybean isolines were examined microscopically 10 days after inoculation with either overcoming strain 94 or nonovercoming strain 110. Although curled root hairs, some with infection threads, were observed by light microscopy on Clark soybean inoculated with either strain 110 or 94, no infection threads and no curled root hairs of the type associated with infection were ever found on Clark-$\underline{rj}_1$ root surfaces. Curled root hairs were seen on surfaces of a majority of the nodules from Clark but not on nodule surfaces from Clark-$\underline{rj}_1$. Curled root hairs were observed by scanning electron microscopy on Clark roots that resembled those with infection threads visualized by light microscopy. Similar curled root hairs were never seen on uninoculated plants or on inoculated Clark-$\underline{rj}_1$.

Several lines of evidence for a possible alternative infection process for the overcoming strains of R. japonicum are suggested. When very high inoculum concentrations of an overcoming strain were used with Clark-$\underline{rj}_1$, apparent

epidermal cell perforation was observed. This implies that the bacteria had the potential to degrade the plant surface directly, and it seems that this might provide an infection pathway. Other evidence of the potential for the overcoming strain's ability to degrade plant surfaces was provided by the roughening of Clark roots by the overcoming strain which was not noted when Clark was inoculated with the nonovercoming strain. Overcoming strains, unlike nonovercoming strains, produced a few nodules in the region of the root which had well developed root hairs at the time of inoculation, suggesting an alternative pathway for infection that is not developmentally limited.

Vincent (1970) described initiation of legume-Rhizobium symbioses in terms of discrete interaction phenotypes. The interaction of Clark with both overcoming and nonovercoming strains would be described as Roc^+, Roa^+, Hac^+, Inf^+, Nod^+, and Fix^+. That is, roots are colonized, root adsorption of rhizobia occurs, root hair curling results, infection threads are formed, leading to nodulation, and nodules are capable of nitrogen fixation. The Clark-rj_1 is Roc^+, Roa^+, Hac^-, Inf^-, Nod^-, Fix^-; demonstrating the expected pattern for an interaction with a block at one of the steps. The interaction is progressive so it is generally assumed that if one is blocked the subsequent phenotypes will be null. The interaction phenotype of Clark-rj_1 with overcoming strains, by this reckoning, is an anomaly; Roc^+, Roa^+, Hac^-, Inf^-, Nod^+, Fix^+. This is the first report of such an interaction phenotype for soybean. The hypothesis that an

additional infection mechanism is present in these combinations is consistent with this reaction type.

APPENDIX A
PLASMIDS OF *RHIZOBIUM JAPONICUM* STRAINS THAT NODULATE SOYBEAN ISOLINES DIFFERING AT THE Rj_1 LOCUS

Plasmids of fast-growing strains of *Rhizobium* carry genes essential for nodulation, including genes affecting host range, and genes that encode enzymes for nitrogen fixation (Sadowsky and Bohlool 1983, Long 1984). These symbiotic functions are generally carried on the very large plasmid usually found in these strains (Casse et al. 1979, Rosenberg et al. 1982). Less is known about the plasmids and their functions in the slow-growing strains of *R. japonicum*. The studies in which techniques were used that produce point mutation do not lend themselves to analysis of plasmid involvement, because adequate genetic markers to which linkage can be measured have not been described for *R. japonicum* plasmids (Maier and Brill 1976, Stacey et al. 1982). The plasmid profiles of a few slow-growing strains of *R. japonicum* have been reported, and some strain-to-strain similarities were noted (Masterson et al. 1982). The results from most studies of *R. japonicum* plasmid content have not shown the similarities reported in that study (Haugland and Verma 1981, Cantrell et al. 1982). Neither are the similarities seen between slow growing strains that are apparent between plasmid profiles for strains of some species of the fast-growing rhizobia

(Broughton et al. 1984), nor indeed, even the similarities seen between strains of the fast growing rhizobia from soybean (Sadowsky and Bohlool 1983, Broughton et al. 1984, Heron and Pueppke 1984).

Certain strains of R. japonicum, referred to as the "overcoming strains," have the ability to form a few nodules on lines of soybean that carry the genotype rj_1rj_1, which is resistant to nodulation by other strains of rhizobia. The objectives of this study were to examine some overcoming strains of R. japonicum for plasmid content, including determination of whether they harbor very large plasmids (> 300 megadaltons, the so-called "megaplasmids"), and whether there is a correlation between the ability of a strain to nodulate rj_1rj_1-soybean and that strain's plasmid profile. The overcoming strains of R. japonicum used were USDA 61, 84, 85, 94, 117, and 119; USDA 110 was used as a non-overcoming control. The bacterial strains used for plasmid molecular weight standards were those reported by Heron and Pueppke (1984) with the additional strain R. japonicum 14C carrying plasmids of 49, 74, 91, and 118 megadaltons (Gross et al. 1979) obtained from A. K. Vidaver, University of Nebraska, Lincoln.

Plasmids were visualized by a modification, similar to that reported by Heron and Pueppke (1984), of the in-gel lysis procedure described by Eckhardt (1978) for electrophoretic detection of bacterial plasmids. The bacteria were cultured to mid-log phase at 27 C with shaking

in liquid defined gluconate-mannitol medium (Bhuvaneswari et al. 1977). For each strain a volume of culture equivalent to 1 x 10^9 cells (determined turbidimetrically) was placed in a Corex centrifuge tube. The subsequent steps were carried out at 4 C. All centrifugations were for 10 min at 7500 x g. R. japonicum strains were washed in 5-10 ml of TE (50 mM Tris, 20 mM EDTA; pH 8) containing 3.0% (w/v) NaCl and 1.0% (w/v) Sarkosyl, followed by centrifugation. Cells were washed in 5-10 ml of TE containing 1.0% Sarkosyl and centrifuged. The pelleted cells were carefully suspended in 2 ml of TE by shaking the tubes gently on a Vortex shaker and then sedimented by centrifugation. Bacteria other than R. japonicum were treated similarly except that the first wash was deleted and the subsequent wash contained 0.1% Sarkosyl. Immediately after the centrifugation following the last wash, the liquid was decanted from the tubes, excess liquid was removed with an adsorbant paper swab, and the tubes were placed on ice. Cells were resuspended in 250 ul of lysis mixture (Heron and Pueppke 1984) by slow shaking on a Vortex shaker. A 25 ul aliquot was immediately transferred to a 10-mm well formed in a 0.7% agarose gel (DNA grade agarose, Sigma Chemical Co., St. Louis, MO; gel apparatus, 160 x 140 x 3 mm, BioRad, Richmond, CA) in TBE (89 mM Tris, 2.5 mM EDTA, 8.9 mM borate; pH 8.2). After 10 min, the cell lysis mixture was overlaid with 25 ul of TBE containing 10% Ficoll 400 (Pharmacia, Uppsala, Sweden) and sodium dodecyl sulfate (SDS) and then with 100 ul of TBE

containing 2.5% Ficoll and SDS. The SDS concentration in the overlay solutions for R. japonicum strains was 1.0%, and 0.1% for all other strains. The wells were sealed with molten 0.7% agarose in TBE. Electrophoresis was carried out in TBE buffer at a constant current of 8 mA for 30 min and then 15 mA until the bromphenol blue marker dye reached the bottom edge of the gel (8-12 hr). Gels were stained in aqueous ethidium bromide (5 ug/l) and were visualized and photographed with transmitted UV light (Maniatis et al. 1982). A photograph of a representative gel is provided in Figure A.1. The logarithms of the reported molecular weights of plasmids from strains used as standards were plotted against the migration distance of those plasmids on the gel. This plot was used to estimate molecular weights of unknown plasmids by calculating the log molecular weight for their migration distance. Estimated molecular weights are expressed as the mean value from 4 to 9 independent observations per strain. The sizes estimated for plasmids are given in Table A.1.

Two strains described here, USDA 110 (= 3I1b110) and USDA 94 (= 3I1b94), also were examined by Masterson et al. (1982). They reported two plasmids of 58 and 118 megadaltons in strain USDA 94. The 118 megadalton plasmid was obtained consistently in this study, but the 58 megadalton plasmid was never observed. Masterson et al. (1982) also detected a 184 megadalton plasmid in strain USDA 110. In this study no plasmid was visualized for strain USDA 110, even though that strain was included on more than

15 gels on which plasmids were clearly evident for other strains. Strain USDA 110 is reported not to have plasmids by Haugland and Verma (1981), and by Cantrell et al. (1982). Both Gross et al. (1979) and Cantrell et al. (1982) reported a plasmid in USDA 117 but none was observed in this study.

No correlation was observed between plasmid content and the ability of these strains to nodulate Clark-*rj*$_1$ soybean. The overcoming strains USDA 61, 85, and 94, each had one plasmid and USDA 84 had two. The overcoming strains USDA 117 and 119, like the nonovercoming strain USDA 110, had none. Although the lack of plasmids has been correlated with the presence of a functional hydrogen uptake system (Cantrell et al. 1982), the plasmids of *R. japonicum* remain cryptic with no fuctions yet associated with their presence.

Figure A.1. Agarose gel electrophoresis of plasmid DNA from strains of Rhizobium japonicum. Lanes A through I are, respectively, R. japonicum strain 14C and strains USDA 61, 84, 85, 94, 84, 110, 117, and 119. Agrobacterium tumefaciens strain C58 is in lane J. Numbers in the margin represent the size of the reference plasmids in megadaltons.

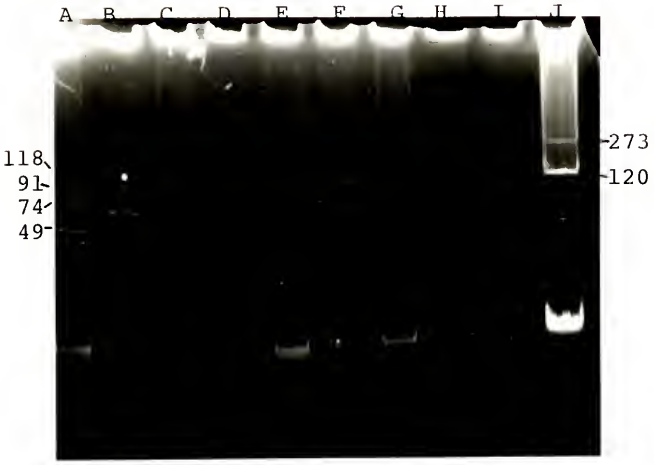

A
B
C
D
E
F
G
H
I
J
118
91
74
49
273
120

Table A.1. Plasmids from strains of *Rhizobium japonicum*

Strain	Molecular mass[a] (megadaltons)
USDA 61	61 ± 4
USDA 84	97 ± 9 153 ± 23
USDA 85	110 ± 17
USDA 94	118 ± 10
USDA 110	NP[b]
USDA 117	NP
USDA 119	NP

[a] Molecular mass is expressed as the mean value ± SD for 4 to 9 independent observations per plasmid.

[b] For strains denoted "NP," no bands characteristic of plasmids were present on at least 15 gels on which plasmids were observed in other strains.

APPENDIX B
EVALUATION OF PROCEDURES REPORTED TO INDUCE HIGH-FREQUENCY MUTATION IN STRAINS OF *RHIZOBIUM JAPONICUM*

The results of preliminary research designed to evaluate a report (Skogen-Hagenson and Atherly 1983) of techniques for high-frequency mutation in strains of *Rhizobium japonicum* are presented here. When this study was undertaken, the literature outlining the role of plasmids in the fast-growing *Rhizobium* species was developing rapidly, but little was known of the role of plasmids in symbiotic functions of *R. japonicum*. The report of a method for inducing high frequency mutations in nodulation and nitrogen fixation genes (Skogen-Hagenson and Atherly 1983) led to evaluation of similar methods for induction of nodulation mutants in the overcoming strains of *R. japonicum*. The overcoming strains produce a few nodules on plants of an isoline of the soybean cultivar Clark that has the nodulation restrictive genotype, $\underline{rj_1}\underline{rj_1}$ (Chapter Three, Devine and Breithaupt 1980). This isoline will be referred to as Clark-$\underline{rj_1}$. The objectives of this study were *i.* to determine whether procedures reported to induce plasmid loss or deletion mutations also produce nodulation mutants in the overcoming strains of *R. japonicum*, and *ii.* to test the usefulness of prescreening mutagenized rhizobia for lack of

homology to *nif* sequences as a means of enriching populations for nodulation mutants.

Modifications of the procedures of Skogen-Hagenson and Atherly (1983) were tested. Bacteria were cultured at elevated temperature with either SDS or ethidium bromide added to the bacterial growth medium. In the first procedure 10^6 cells of *R. japonicum* strain 94 were added per ml of liquid gluconate-mannitol (G-M) medium (Bhuvaneswari et al. 1977) containing various concentrations of SDS (Table B.1). The cultures were incubated at 37 C in the dark without shaking for 32 d and then were diluted and plated on yeast extract-mannitol agar (YEM) (Vincent 1970). One hundred colonies were selected at random, plated in duplicate, and each was inoculated onto seedlings in a plastic growth pouch with 2 seedlings in each of either Clark or Clark-*rj*$_1$. The seedlings had been grown for one day in plastic growth pouches from seed disinfested and germinated as described in Chapter Three. Each colony was scraped from YEM with the broad end of a flat, sterile toothpick and suspended in 1 ml of sterile water. Bacteria from each of the duplicate plates were inoculated onto plants of one of the isolines. The bacterial suspension was dripped over the roots of the plants in a pouch with a sterile pipette. Inoculated plants were grown at an ambient temperature of about 27 C under fluorescent lights with a 12 hr on-off cycle at an irradiance of 450 uE/m^2/sec (400-700 nm). Plants were screened for nodulation at approximately

Table B.1. Survival of Rhizobium japonicum strain USDA 94 after 32 days at 37 C in gluconate-mannitol medium amended with sodium dodecyl sulfate (SDS).

SDS (%)	Colony forming units / ml
0.00	7.7×10^3 [a]
0.01	3.1×10^4
0.10	1.2×10^4

[a] The initial bacterial concentration was 10^6 cells/ml. Cultures were kept in the dark without shaking.

2 wk after inoculation and subsequently at weekly intervals up to 30 d. Strains inoculated on Clark were scored as nonnodulating (0 nodules per pouch after 30 d) or nodulating (1 or more nodules formed within 30 d). Strains inoculated on Clark-*rj*$_1$ were rated for enhanced nodulating ability (arbitrarily designated 2 or more nodules per plant) at 30 d. Any strains rated nonnodulating on Clark or rated for enhanced nodulating ability on Clark-*rj*$_1$ were retested by inoculating 5 growth pouches, each with 10 plants of the appropriate host.

In addition to the strains rated directly on plants for nodulation, 75 colonies were selected at random for colony-hybridization preselection. Colonies were plated in a 5 x 5 array on YEM agar in standard 100 mm dia plates. Colony hybridization was carried out using 95 mm dia circles of 0.45 um nitrocellulose filter (Schleicher & Schuell, Keen, NH) following the procedures outlined by Maniatis et al. (1982 [p. 326]) for binding DNA from bacterial colonies to nitrocellulose. The nitrocellulose filters were probed with *nif*HDK sequences cloned from *Klebsiella pneumoniae* (Ruvkin and Ausubel 1980). *Escherichia coli* strain HB101 containing the cloned *nif* genes in plasmid pACYC184 as recombinant plasmid pSA30, was obtained courtesy of K. T. Shanmugam, Department of Microbiology and Cell Science, University of Florida, Gainesville. The *nif* sequence was purified by standard procedures (Maniatis et al. 1982) for plasmid purification, restriction, gel electrophoresis, and recovery on activated DEAE-cellulose. The probe was labelled with

cytidine 5'-[^{32}P]-triphosphate (Amersham Corp. Arlington Heights, IL) using nick translation (Maniatis et al. 1982 [p. 109]). Hybridization of the probe to the nitrocellulose filters bearing the colony replicas as bound DNA was accomplished by the procedure described in Maniatis et al. (1982 [p. 326]) using a hybridization temperature of 68 C for 24 hr. Colonies identified by reduced binding of the nifHDK probe in colony hybridization were cultured individually in 50 ml of liquid G-M medium, harvested and inoculated on seedlings grown in pouches as described in Chapter Three. A total of 10 plants in 5 pouches were rated for each putative mutant screened. Plants were rated for ability of the bacteria to nodulate and for evidence of nitrogen fixation. Nitrogen fixation was assumed to be efficient if the plant was a healthy green color and nodules were large and had leghemoglobin (on the basis of internal red color on visual examination).

The second experiment for high-frequency induction of mutation was as described by Skogen-Hagenson and Atherly (1983). The media used were YEM and the Skogen-Hagenson and Atherly medium (SHAM), which is a yeast-extract medium containing high iron. R. japonicum strains USDA 74 and 76 were cultured in the dark at 36 C with shaking in each of the media amended with ethidium bromide at 50 ug/ml. Control flasks included the 2 strains in each of the unamended media grown at the treatment temperature, and the 2 strains in amended media grown at 28 C. Every 4 d an aliquot was

removed from each flask, diluted, and plated for determination of colony forming units. Two-hundred-fifty colonies were tested for ability to nodulate Clark; 100 of those colonies also were tested for enhanced ability to nodulate Clark-*rj*$_1$. The tested bacteria were from colonies of strain USDA 76 selected from dilution plates of cells removed from the amended SHAM treatment on day 10.

As seen in Table B.1 the survival rate of *R. japonicum* strain 94 is high after 32 d in SDS-amended G-M medium incubated at 37 C. The 100 colonies selected from the SDS treatment nodulated Clark soybean and none produced two or more nodules per plant on Clark-*rj*$_1$. Of the 75 colonies hybridized to the *nif* probe, 7 were selected on the basis of greatly reduced hybridization and were screened on plants. All nodulated both Clark and Clark-*rj*$_1$ soybean with no obvious alteration in nodulation pattern. Five of those strains appeared to have normal nitrogen-fixation phenotype, but two produced smaller nodules which had white-green centers and the plants showed symptoms consistent with nitrogen difficiency.

Figure B.1 represents the survival curves for the strains USDA 74 and 76 in both YEM and SHAM, each amended with ethidium bromide at 50 ug/ml. The rates of bacterial death are approximately the same as reported by Skogen-Hagenson and Atherly (1983) for strains USDA 74 and 61A76 in similarly amended SHAM. No changes in nodulation phenotype were detected in the 250 selections of treated USDA 76 on Clark or the 100 of those tested on Clark-*rj*$_1$.

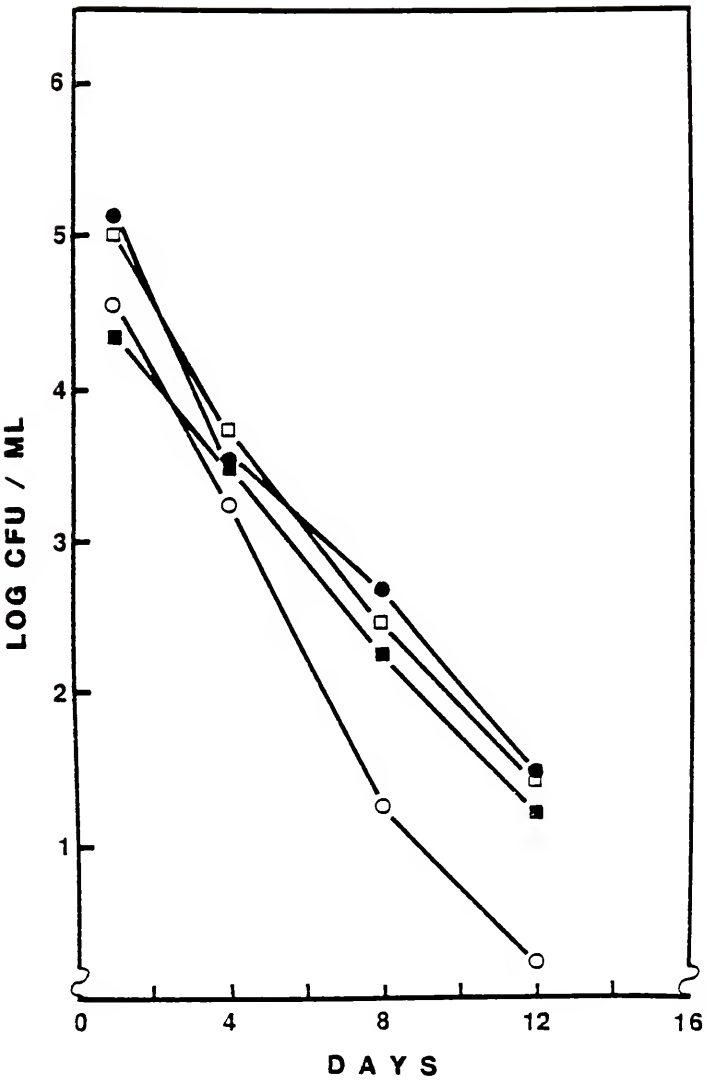


Figure B.1. Survival curves for Rhizobium japonicum strains in media amended with ethidium bromide at 50 ug/ml. ●-strain 76 in Skogen-Hagenson and Atherly (1983) medium (SHAM), ■-strain 76 in yeast extract-mannitol (YEM) medium (Vincent 1977), ○-strain 74 in SHAM, □-strain 74 in YEM.

The number of putative mutants screened for lack of binding to K. pneumoniae nif sequences and tested for nodulation are inadequate to demonstrate that nif and nodulation genes are not linked in these strains, although the limited data are consistent with the apparent lack of linkage in R. japonicum (Hennecke 1981, Fuhrmann and Hennecke 1983, 1984). Given these results and the growing evidence for lack of linkage of these functions this method for enrichment of Nod$^-$ mutants of slow-growing R. japonicum seems not to have the utility that it has for the fast-growing rhizobia (Hirsch et al. 1982, 1984).

The use of heat and curing agents did not induce nodulation mutants in high frequency; none of 350 isolates selected from treatments reported to induce high-frequency mutation in symbiotic functions showed an obvious change in nodulation phenotype. This contrasts sharply with the previous report that 44 of 133 isolates (33%) were Nod$^-$ (Skogen-Hagenson and Atherly 1983). Classical mutagenesis with chemical agents yielded 2 nodulation mutants in R. japonicum out of 2500 isolates tested (Maier and Brill 1976). Hom et al. (1984) reported no Nod$^-$ mutants out of 200 isolates carrying Tn5. Although these procedures require extensive screening of isolates on plants and produce mutants at low frequencies, the apparent randomness of mutations produced, rather than tending to be specific for plasmid encoded genes, make them more suitable for screening for production of nodulation mutants in R. japonicum. The use of transposon-induced mutation in

particular holds promise because of the ability to use positive selection for introduced markers *in vivo* and for homology to introduced DNA sequences *in vitro*.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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